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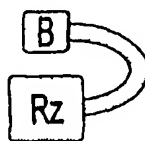
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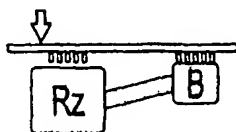
(54) Title: A TARGET-DEPENDENT NUCLEIC ACID ADAPTER

OFF conformation
(Inactive)



RNA target

ON conformation
(Active)



(57) Abstract: In accordance with the present invention, there is provided a nucleic acid target-dependent adapter linked to a nucleic acid sequence. The adapter comprises linked together a biosensor having a specific sequence complementary to a target sequence of a substrate, the biosensor improving the specificity of the nucleic acid sequence for the substrate, and a blocker stem sequence complementary to a portion of the nucleic acid sequence. In absence of the target sequence of the substrate, the blocker stem sequence forms an intramolecular stem with the nucleic acid sequence linked thereto, preventing exposition of the sequence of the nucleic acid sequence, thus locking the nucleic acid sequence so linked to the adapter in an inactive conformation, and in presence of the target sequence, the blocker stem sequence dissociating from the nucleic acid sequence, thus exposing the nucleic acid sequence linked to the adapter in an active conformation.

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A TARGET-DEPENDENT NUCLEIC ACID ADAPTER

TECHNICAL FIELD

The present invention relates to the field of target-dependent switch adapters for nucleic acid sequences, and more particularly to adapters for
5 nucleic acid sequences such as ribozymes.

BACKGROUND OF THE INVENTION

Discoveries in the basic realm of molecular biology over the past ten years have led to the realization that RNA has a series of distinct capabilities and biological activities previously unsuspected. The most important of these
10 novel RNA-level discoveries has been the finding that RNA can be an enzyme as well as an information carrier.

Various RNA molecules have one or more enzymatic activities, such as an endoribonuclease activity which acts to cleave other RNA molecules. Such activity is termed intermolecular cleaving activity. These enzymatic RNA
15 molecules are derived from an RNA molecule which has an activity which results in its own cleavage and splicing. Such self-cleavage is an example of an intramolecular cleaving activity.

Since 1982, several unexpected diseases caused by RNA-based pathogenic agents have emerged. These include the lethal Acquired Immune
20 Deficiency Syndrome (AIDS) and *delta* hepatitis (also called Hepatitis D), a particularly virulent form of fulminant hepatitis caused by a viroid-like RNA agent. These blood-borne diseases are spread at the RNA level, manifest

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themselves in cells of patients, and are by now present within the bloodstream of millions of individuals.

Conventional biotechnology, with its reliance on recombinant DNA methods and DNA-level intervention schemes, has been slow to provide valid
5 approaches to combat these diseases.

The potential of ribozymes (RNA enzymes) to catalyze the cleavage of RNA substrates makes them attractive molecular tools. Ribozymes are an interesting alternative to RNA interference approach that seems to trigger immunological responses. Many efforts were directed at increasing the
10 substrate specificity of ribozyme cleavage, which can be considered as a limit to their utilization. For example, allosteric ribozymes for which the catalytic activity is regulated by an independent effector, have been developed.

Delta ribozymes, derived from the genome of hepatitis *delta* virus (HDV), are metalloenzymes. Like other catalytically active ribozymes, namely
15 hammerhead and hairpin ribozymes, the *delta* ribozymes cleave a phosphodiester bond of their RNA substrates and give rise to reaction products containing a 5'-hydroxyl and a 2',3'-cyclic phosphate termini. Two forms of *delta* ribozymes, namely genomic and antigenomic, were derived and referred to by the polarity of HDV genome from which the ribozyme was generated. Both HDV
20 strands forms exhibit self-cleavage activity, and it has been suggested that they are involved in the process of viral replication. This type of activity is described as *cis*-acting *delta* ribozymes.

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Like other ribozymes, *delta* ribozymes have a potential application in gene therapy in which an engineered ribozyme is directed to inhibit gene expression by targeting either a specific mRNA or viral RNA molecule. A very low concentration (< 0.1 mM) of Ca^{2+} and Mg^{2+} is required for *delta* ribozyme
5 cleavage.

With respect to the structure of the δ ribozyme, it folds into a compact secondary structure that includes pseudoknots (for reviews see Bergeron et al., *Current Med. Chem.* 10, 2589-2597, 2003). This structure is composed of one stem (the P1 stem), one pseudoknot (the P2 stem is a pseudoknot in the *cis-*
10 acting version), two stem-loops (P3-L3 and P4-L4) and three single-stranded junctions (J1/2, J1/4 and J4/2). Both the J1/4 junction and the L3 loop are single-stranded in the initial stages of folding, but are subsequently involved in the formation of a second pseudoknot that consists of two Watson-Crick base pairs (the P1.1 stem). In terms of general organization, the P1 and P3 stems,
15 along with the J4/2 junction, form the catalytic center, while the P2 and P4 stems are located on either side of the catalytic centre and stabilize the overall structure.

The binding domain of δRz (the P1 stem) is composed of one G-U wobble base pair followed by six Watson-Crick base pairs. In addition, the
20 nucleotides from position -1 to -4 of the substrate, that is those adjacent to the scissile phosphate, were shown to contribute to the ability of a substrate to be cleaved efficiently. Thus, the substrate specificity of δRz cleavage is based on a total of 11 nucleotides, which might be a limiting factor when trying to specifically target an RNA species in a cell. Because the P1 stem is located

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within its catalytic center, all attempts to modify the length of this stem result in the loss of catalytic ability.

In International publication WO99/55856 (Jean-Pierre Perreault et al.), the entire content of which is hereby incorporated by reference, filed in the
5 name of Université de Sherbrooke, there is disclosed a nucleic acid enzyme for RNA cleavage, and more particularly a *delta* ribozyme and mutants thereof.

In United States Patent No. 5,225,337 (Hugh D. Robertson et al.), issued on July 6, 1993, there are disclosed ribozymes derived from a specific domain present in the hepatitis *delta* virus (HDV) RNA for specifically cleaving targeted
10 RNA sequences and uses thereof for the treatment of disease conditions which involve RNA expression, such as AIDS. These ribozymes consist in at least 18 consecutive nucleotides from the conserved region of the hepatitis *delta* virus between residues 611 and 771 on the genomic strand and between residues 845 and 980 on the complementary anti-genomic strand. These ribozymes are
15 proposed to fold into an axe-head model secondary structure. According to this model structure, these ribozymes require substrate base paired by 12-15 nucleotides. More specifically, a substrate bound to the ribozyme through the formation of two helices. A helix is located upstream to the cleavage site (i.e. in 5' position) while the second helix is located downstream to the cleavage site
20 (i.e. in 3' position).

In United States Patent No. 5,625,047 (Michael D. Been et al.), issued on April 29, 1997, there are disclosed enzymatic RNA molecules proposed to fold into a pseudoknot model secondary structure. These ribozymes were proposed to cleave at almost any 7 or 8 nucleotide site having only a preference for a

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guanosine base immediately 3' to the cleavage site, a preference for U, C or A immediately 5' to the cleavage site, and the availability of a 2' hydroxyl group for cleavage to occur. The specificity of recognition of these ribozymes is limited to 6 or 7 base pairing nucleotides with the substrate and a preference of the first 5 nucleotide located 5' to the cleavage site. Neither tertiary interaction(s) between the base paired nucleotides and another region of the ribozyme, nor single-stranded nucleotides are involved to define the specificity of recognition of these ribozymes. Because the recognition features were included in a very small domain (i.e. 6 or 7 base paired nucleotides) in order to exhibit the desired 10 activity, these ribozymes have a limited specificity, and thus, not practical for further clinical applications.

It would be highly desirable to be provided with a new ribonucleic acid, target-dependent adapter to increase specificity of the nucleic acid for its target.

SUMMARY OF THE INVENTION

15 One aim of the present invention is to provide a new ribonucleic acid, target-dependent adapter to increase efficiency and specificity (prevents cleavage of an inappropriate target) of cleavage of a ribonucleic acid for its target. Moreover, the adapter of the present invention can be used as a switch to turn *on* or *off* a ribonucleic acid enzyme by controlling availability of the target 20 of this enzyme. Whenever the target is not available, the adapter turns *off* the enzyme by adopting an inactive conformation and when the substrate or the target is detected by the adapter, the enzyme is turned *on* in an active conformation. The same principle in the present invention is also applicable to any nucleic acid, where such nucleic acid when linked to the adapter would be

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hidden (in an inactive conformation) or prevented to react with anything else in absence of a target and would be made available to react with its target upon detection of said target by the adapter.

It is reported herein below a new switch made by molecular engineering of a ribozyme, possessing a biosensor module that switches the cleavage from *off* to *on* in the presence of the target substrate. Both proof-of-concept and mechanism of action of this man-made switch are reported herein below using a modified hepatitis *delta* virus ribozyme that can cleave RNA transcripts derived from both the hepatitis B and C viruses. This new approach provides a highly specific and improved tool for functional genomics and gene therapy. In fact, the same modifications made to the Hepatitis Delta Virus ribozyme can be made to other ribozymes as well as other RNA- and DNA-based approaches. Moreover, the switch of the present invention can be modified to be adapted to any nucleic acid sequences that target a substrate, making the switch a new versatile and powerful tool, allowing to increase the specificity of the nucleic acid for its substrate or target, also allowing to increase the cleavage efficacy of the ribozyme for its substrate or target, and to abolish the non-specific pairing therefore reducing false positive reactions.

In accordance with the present invention there is provided a target-dependent nucleic acid adapter adapted to be matched to a substrate comprising a target sequence, said adapter having a nucleic acid sequence comprising linked together:

- i) a blocker stem sequence complementary to a portion of said nucleic acid sequence; and

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- ii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the nucleic acid sequence for said target sequence,

wherein in absence of the target sequence of said substrate, said blocker stem
5 sequence forms an intramolecular stem with said nucleic acid sequence linked thereto, preventing exposition of the nucleic acid sequence, thus locking said nucleic acid sequence of the adapter in an inactive conformation, and, in presence of said target sequence of said substrate, said biosensor sequence forming conventional Watson-Crick base pairs with said target sequence and
10 said blocker stem sequence dissociating from the intramolecular stem, thus exposing said nucleic acid sequence of said adapter in an active conformation.

The target dependent nucleic acid adapter may also comprises sequences forming a stabilizing stem, whereby the 3'-end of the adapter so linked to said nucleic acid sequence is paired up, thus preventing or reducing
15 degradation of said nucleic acid sequence. The stabilizing stem may have for example two complementary strands, a first strand of which is linked to the 5'-end of the biosensor, and a second strand of which that is complementary to the first strand and that is adapted to be linked at its 5'-end to the 3'-end of the nucleic acid sequence, thus preventing exposure of a single stranded 3'-end
20 sequence susceptible to degradation by cellular nuclease.

In one embodiment of the invention, the first strand of the stabilizing stem has a sequence as set forth from residue 4 to 11 of SEQ ID NO:1 and the second strand of the stabilizing stem has a sequence as set forth from residue 96 to 103 of SEQ ID NO:1.

Still in one embodiment of the invention, the blocker stem sequence has a sequence specific for a ribozyme, such as ribozyme *delta*.

In a further embodiment of the invention, the biosensor has a sequence as set forth from residue 15 to 29 of SEQ ID NO:1. The blocker stem sequence
5 has in one embodiment a sequence as set forth from residue 30 to 33 of SEQ ID NO:1. Preferably, the blocker stem sequence is linked downstream of the biosensor.

Still in accordance with the present invention, there is provided a method for improving specificity of a nucleic acid sequence for a target sequence, said
10 method comprising the steps of attaching to said nucleic acid sequence a target-dependent nucleic acid adapter having a nucleic acid sequence comprising:

- i) a blocker stem sequence complementary to a portion of said nucleic acid sequence; and
- 15 ii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the nucleic acid sequence for said target sequence,

wherein in absence of the target sequence of said substrate, said blocker stem sequence forms an intramolecular stem with said nucleic acid sequence linked
20 thereto, preventing exposition of the nucleic acid sequence, thus locking said nucleic acid sequence of the adapter in an inactive conformation, and, in presence of said target sequence of said substrate, said biosensor sequence forming conventional Watson-Crick base pairs with said target sequence and

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said blocker stem sequence dissociating from the intramolecular stem, thus exposing said nucleic acid sequence of said adapter in an active conformation.

Further in accordance with the present invention, there is provided a method for turning *on* or *off* an enzymatic activity of a nucleic acid molecule having an enzymatic activity, said method comprising the steps of attaching to
5 said nucleic acid molecule a nucleic acid target dependent adapter having a nucleic acid sequence comprising:

- i) a blocker stem sequence complementary to a portion of said nucleic acid sequence; and
- 10 ii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the nucleic acid sequence for said target sequence,

wherein in absence of the target sequence of said substrate, said blocker stem sequence forms an intramolecular stem with said nucleic acid sequence linked thereto, preventing exposition of the nucleic acid sequence, thus locking said
15 nucleic acid sequence of the adapter in an inactive conformation, turning off the enzymatic activity and, in presence of said target sequence of said substrate, said biosensor sequence forming conventional Watson-Crick base pairs with said target sequence and said blocker stem sequence dissociating from the
20 intramolecular stem, thus exposing said nucleic acid sequence of said adapter in an active conformation, turning on the enzymatic activity.

In accordance with one embodiment of the present invention, there is also provided a target-specific activatable/deactivatable ribonuclease adapted

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to be matched to a substrate comprising a target sequence, said ribonuclease having a nucleic acid sequence comprising linked together:

- i) a ribonuclease sequence, or an active fragment thereof;
- ii) a blocker stem sequence complementary to a portion of said
5 ribonuclease sequence, said blocker sequence being linked upstream of the ribonuclease sequence; and
- iii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the ribonucleic acid sequence for said target sequence, said biosensor being
10 linked to the blocker sequence,

wherein in absence of the target sequence of said substrate, said blocker sequence forms an intramolecular stem with the ribonuclease sequence linked thereto, thus locking said ribonuclease in an inactive conformation, and, in presence of the target sequence of said substrate, said biosensor sequence
15 forming conventional Watson-Crick base pairs with said target sequence and said blocker stem sequence dissociating from the intramolecular stem, thus exposing said ribonuclease in an active conformation.

In a further embodiment of the invention, the target-specific activatable/deactivatable ribonuclease has a sequence as set forth in SEQ ID
20 NO:1.

For the purpose of the present invention the following terms are defined below.

The term "RNA with enzymatic or effector activity" is intended to mean any RNA that has an active and inactive conformation or any RNA that has an enzymatic activity or that has an effect on either the transcription of said target RNA or a downstream event following transcription of said RNA.

- 5 The term "substrate" can be substituted by "target" or "target substrate" or the expression "substrate or target" throughout the application. It is to be recognized and understood that the substrate contains a target sequence.

The term "adapter" can be substituted by the term "switch" throughout the application.

- 10 The term "Biosensor" can be abbreviated as "BS" or "BSO"

The term "SOFA module" can be substituted by the term "SOFA adapter" throughout the application.

The term "Target dependant nucleic acid adapter" can be substituted by the term "nucleic acid target dependent adapter" throughout the application.

- 15 In accordance with one embodiment of the present invention, there is provided a method for turning *on/off* an enzymatic activity of a nucleic acid molecule having an enzymatic activity. The target-specific activatable/deactivatable adapter can be adapted to any type of nucleic acid enzymes catalyzing the modification of nucleic acid substrates (i.e. modifying
20 enzymes such as kinases, ligases, methylases, ribonucleases, aminoacyl-tRNA synthetases, etc).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of both the *off* and *on* conformations of the SOFA-ribozyme of the present invention;

5 Figs. 2A and 2B illustrate the specific structure and sequence of the SOFA-ribozyme (SEQ ID NO:1), in the *off* (Fig. 2A) and *on* (Fig. 2B) conformations, in accordance with one embodiment of the present invention, where the arrow in the *on* conformation indicates the cleavage site on the target (SEQ ID NO:2);

10 Fig. 3 illustrates an autoradiogram of a denaturing 6% PAGE gel for the analysis of the cleavage reaction of the HBV-derived target by the original- (Wild Type) (WT), SOFA- δ Rz-303 and SOFA- δ Rz-513 ribozymes;

Fig. 4 illustrates a graphical representation of time courses for the cleavage reactions of SOFA⁺ (squares), SOFA⁻ (circles) and the original (inversed triangles) ribozyme versions of δ Rz-303 (filled) and δ Rz-513 (empty);

15 Fig. 5 illustrates an autoradiogram of a denaturing 6% PAGE gel showing the cleavage assays of the HBV-derived substrate by SOFA- δ Rz-303 bearing a biosensor stem of various lengths (BS-X, where X indicates the length of the stem) to characterize the SOFA- δ Rz-303;

20 Fig. 6 illustrates an autoradiogram of a 20% PAGE gel showing the cleavage assays of the HBV-derived substrate of 44 nucleotides by a SOFA- δ Rz-303 bearing a biosensor stem of various lengths (BS-X, where X indicates the length of the stem);

Figs. 7A and 7B illustrate an analysis of the mechanism of action of the SOFA-ribozyme, showing the proposed sequential interactions between the ribozyme and the substrate (Fig. 7A), and the relative cleavage efficiencies calculated from two independent sets of experiments using the original- (WT),
5 SOFA⁺- and SOFA⁻- δ Rz-303 ribozymes incubated either with or without the FCO, BSO and unrelated (UNO) oligodeoxyribonucleotides (Fig. 7B);

Figs. 8A to 8F illustrate an analysis of the substrate specificity of various SOFA-ribozymes, where Figs. 8A to 8C show a schematic representation of the various substrates, while Figs. 8D to 8F illustrate the autoradiograms of
10 denaturing 6% or 20% PAGE gels performed for these cleavage assays;

Fig. 9 illustrates the sequences of all the targeting sites used in Figs. 1 to 8 except in Figs. 8A and 8D;

Fig. 10A illustrates the stem formed between 8 pairs of substrate (a to h, left) and ribozyme biosensor (A to H, right) sequences;

15 Fig. 10B and 10C illustrate the autoradiogram of a typical 10% denaturing PAGE gel of a time course experiment performed under single turnover conditions for the pair Dd, and the graphical representation of the time course of ribozyme D cleaving each of the substrates (a to h);

Fig. 10D illustrates the histogram of the k_{obs} values for each of the 64
20 possible pairs;

Fig. 11A illustrates twenty-three biosensor sequence variants examined for their ability to cleave the short 44 nt HBV-derived substrate. The mutations

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are boxed in grey, and the k_{obs} values (in min^{-1}) are indicated on the right. The stars indicate the SOFA- δRz -303 mutants for which the k_{cat} and K_{M} values were determined for the cleavage of a long version of the HBV-derived substrate (1190 nt);

5 Fig. 11B illustrates the average values of k_{obs} from at least two independent sets of experiments for each cluster of mutated ribozymes.

Fig. 12A illustrates four blocker stems tested;

Fig. 12B illustrates an autoradiogram of a 6% denaturing PAGE of the
10 cleavage assays performed with the SOFA- δRz -303 variants possessing mutated blocker sequences (i.e. BL-X, where X indicates the size of the blocker stem). The reactions were performed under single turnover conditions using the 1190-HBV substrate. The sizes of the bands are indicated on the right of the gel. The control (-) was performed in the absence of ribozyme;

15 Fig. 12C illustrates a kinetic analysis performed for each of the mutants: BL-0, squares; BL-2, circles; BL-4, inverse triangles; and, BL-5, diamonds.

Fig. 13A illustrates the design of the substrates used to analyze the importance of the spacer sequence. The substrate P1 strand of SOFA- δRz -303
20 was repeated seven times ($\text{P1}_{\text{N}, 1-7}$) within seven substrates possessing spacers of different sizes (0 to 6 nt);

Fig. 13B illustrates an autoradiogram of a 10% denaturing PAGE of the cleavage assays performed with each of the seven substrates. Lanes 0 to 6 correspond to the different sizes of the spacer sequences (i.e. from 0 to 6 nt).
25 The migrations of the substrates (S) and their sizes, as well as those of the

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cleavage products, are indicated adjacent to the gel. XC and BPB indicate xylene cyanol and bromophenol blue;

Fig. 13C illustrates the relative percentage of cleavage as a function of spacer length. The bracket indicates the optimal length (1 to 5 nt), and dashed lines separate the observed transitions;

Fig. 13D illustrates the histogram of the relative percentage of cleavage of the substrates possessing spacers of various lengths (5, 19, 33 and 47 nt). The inset shows the autoradiogram of the corresponding 10% denaturing PAGE gel;

10

Fig. 14A illustrates an autoradiogram of a 6% denaturing PAGE of cleavage assays of various SOFA- δ Rz-303 variants synthesized to evaluate the importance of the stabilizer sequence, where lane 1 is the incubation of the long HBV-derived substrate (1190 nt) alone, while lane 2 is that in the presence of the original δ Rz-303, lanes 3 and 4 are the cleavage assays performed with SOFA⁺- and SOFA⁻- δ Rz-303 including the stabilizer stem, respectively, lanes 5 and 6 are the cleavage assays performed with the SOFA⁺- and SOFA⁻- δ Rz-303 lacking the stabilizer stem, respectively. The sizes of the bands are indicated adjacent to the gel;

Fig. 14B illustrates the result obtained with mutated stabilizer. The upper panel illustrates the sequence of the stabilizer (SOFA- δ Rz-303-ST1 to -ST4), while the lower panel illustrates the autoradiogram of the 6% PAGE of the corresponding cleavage assays. The control (-) was performed in the absence of ribozyme;

Fig. 15A illustrates a schematic representation of the SOFA-ribozyme in both the *off* and *on* conformations, where the *on* conformation is obtained after the addition of the substrate. The bold lines indicate the oligodeoxynucleotides.

Fig. 15B illustrates an autoradiogram of an 8% denaturing PAGE of the
5 probing assay. The symbols (-) and (+) indicate the presence or absence, respectively, of the substrate for the probing performed using each oligodeoxynucleotide (L3', P4', BS', BL' and ST'). The positions of the expected cleavage products, XC and BPB are indicated adjacent to the gel.

Fig. 16A illustrates the expression vector of the HBV-derived gene C
10 used in the *in vivo* cleavage assays of the HBV-derived substrate by SOFA- δ Rz-303;

Fig. 16B illustrates the expression vector for various ribozyme versions in accordance with various embodiments of the present invention, used in the *in vivo* cleavage assays of the HBV-derived substrate by SOFA- δ Rz-303;

15 Fig. 16C illustrates autoradiograms of a Northern blot hybridization performed after a denaturing 1.3% agarose gel where β -actin and HBV mRNAs were detected using ^{32}P -labelled RNA probes;

Figs. 17A, 17B and 17C show sequences and secondary structures of the SOFA+- δ Rz-Down (SOFA+- δ Rz-DN) and SOFA+- δ Rz-Double (SOFA+- δ Rz-
20 DB), demonstrating the versatility of the SOFA- δ Rz-303.

Fig. 17D illustrates autoradiograms of denaturing 6% PAGE gels performed for these cleavage assays, including a control (-) performed in the absence of ribozyme;

Fig. 18A illustrates the sequence and secondary structure of the
5 SOFA⁺-δRz without the stabilizer (SOFA⁺-δRz-NS, NS for no stabilizer);

Fig. 18B illustrates autoradiograms of 6 % denaturing PAGE gel of the cleavage assays;

Fig. 19A to 19F illustrate the sequence and secondary structure of various SOFA-ribozymes and SOFA-DNzyme in accordance with one
10 embodiment of the invention, showing both *off* and *on* conformations, wherein the small arrows of the *on* conformations indicate the cleavage or ligation sites;

Fig. 20 illustrates an autoradiogram of 6 % denaturing PAGE gel of the cleavage assays obtained for the SOFA-DNzyme; and

Figs. 21A to 21C illustrate the sequence of a SiRNA (Fig. 21A), the
15 sequence and secondary structure of a SOFA-siRNA version in accordance with a further embodiment of the invention, showing both *off* (Fig. 21B) and *on* (Fig. 21C) conformations, wherein the small arrows of the *on* conformation indicate potential cleavage sites.

DETAILED DESCRIPTION OF THE INVENTION

20 With the aim of generating highly specific ribozymes that could be regulated by the presence/absence of their target substrates, the inventors started with the concept that a ribozyme should be linked to a target-dependent

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module that acts as a biosensor (Fig. 1). In absence of its target, the ribozyme is inactive (*off*), while in the presence of the desired target the biosensor recognizes it and activates (*turns on*) the ribozyme's cleavage activity. Accordingly, a rational design led to a ribozyme controlled by a novel Specific
5 *On/off* Adapter (SOFA). The original hepatitis *delta* virus (HDV) ribozyme, for which substrate recognition is based solely on the formation of seven base pairs in the P1 stem, was used as a suitable model (Bergeron, L.J., et al., *Current Med. Chem.* **10**, 2589-2597, 2003).

In one embodiment of the invention, the SOFA (Box 8 on Figs. 2A and
10 2B) includes three domains, also called sequences or segments:

- a blocker (Sequence from ribonucleotide 30 to 33 of SEQ ID NO:1 on Figs. 2A and 2B; Box 10,
- a biosensor (BS; Sequence from ribonucleotide 15 to 29 of SEQ ID NO:1 on Figs. 2A and 2B; Box 12) and
- 15 - a stabilizer (Sequence from ribonucleotide 4 to 11 of SEQ ID NO:1 pairing with ribonucleotides 103 to 93 of SEQ ID NO:1, respectively, Figs. 2A and 2B; Box 14).

In the absence of the target (Box 16 on SEQ ID NO:2, Figs. 2A and 2B), the blocker 10 forms an intramolecular stem with the P1 strand (Sequence from
20 ribonucleotide 55 to 61 of Figs. 2A and 2B; Box 18), thereby generating an inactive conformation. Upon the addition of an adequate target, the biosensor anneals with the substrate, thereby releasing the P1 strand so that it can subsequently hybridize with the substrate, initiating formation of the active

conformation. Thus, the target has two roles acting simultaneously, one as an activator and one as a substrate. The biosensor acts as a riboswitch regulating the catalytic activity. Finally, the stabilizer localizes the 3'-end of the SOFA module in a double-stranded region that stabilizes the ribozyme from the cellular
5 nucleases (Lévêque, D., et al., *RNA* 8, 464-477, 2002).

To achieve the aim of the present invention, the inventors developed a switch, also referred to herein as a Specific *On/Off* Adapter or SOFA to improve specificity of a nucleic acid sequence such as DNA or RNA for its target and/or control the activity of said nucleic acid sequence. This construct can be made
10 specific to particular ribozymes or RNA with enzymatic or effector activity, to activate or inactivate ribozymes or RNA simply by changing and matching the sequence of the biosensor with the complement of that of the target sequence, so that pairing up between the two can occur, when in presence of each other.

The biosensor must bind its complementary sequence on the substrate in
15 order to unlock the SOFA module, thereby permitting the folding of the catalytic core into the *on* conformation. Both the blocker and the biosensor have been shown to increase the substrate specificity of the ribozyme's cleavage by several orders of magnitude as compared to the wild-type δ Rz. This is due mainly to the addition of the biosensor domain that increases the binding
20 strength of the δ Rz to its target, but is also due to the fact that the blocker domain interacts with the P1 region and decreases its binding capacity. Finally, the presence of the stabilizer, which has no effect on the cleavage activity, stabilizes the RNA molecule *in vivo* against ribonucleases. The purpose of the stabilizer sequence is to pair up the 3' end of the sequence to prevent

degradation. Both proof-of-concept and the preliminary characterization of SOFA- δ Rz that cleaves RNA transcripts derived from the hepatitis B and C viruses are reported here.

METHODS

5 HBV, HCV and ribozyme DNA constructs

An HBV pregenome insert (from pCHT-9/3091, Nassal, M. *J. Virol.* **66**, 4107-4116, 1992) was subcloned downstream of the T7 RNA promoter in the vector pBlueScript SKTM (Stratagene) using the *Sal*I and *Sac*I restriction sites, and the resulting plasmid named pHBVT7 (Bergeron, L.J., & Perreault, J.P. *Nucleic Acids Res.* **30**, 4682-4691, 2002). An HBV 1190 nt fragment was excised from pCHT9/3091 using *Sac*I and *Eco*RI, and then subcloned into pBlueScript SKTM, generating pHBV-1190. A shorter HBV 44 nt substrate was produced using a PCR-based strategy with T7 sense primer: 5'-TTAATACGAC TCACTATAGG G-3' (SEQ ID NO:3) and antisense primer: 5'-CTTCCAAAAG
15 TGAGACAAGA AATGTGAAAC CACAAGAGTT GCCCTATAGT
GAGTCGTATT AA-3' (SEQ ID NO:4). The plasmid pHCVa was obtained by cloning the 1348 nt HCV 5' sequence from pHCV-1b (Alaoui-Ismaili, et al., *Antiviral Res.* **46**, 181-193, 2000) into *Hind*III and *Bam*HI pre-digested pcDNA3 vector. The original δ ribozymes were constructed as described previously
20 (Bergeron, L.J., & Perreault, J.P. *Nucleic Acids Res.* **30**, 4682-4691, 2002). SOFA^{+/} ribozymes were constructed using a PCR-based strategy including two complementary and overlapping oligonucleotides. Briefly, two DNA oligonucleotides were used: i) the antisense oligonucleotide (Rz-down; 5'-

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CCAGCTAGAA AGGGTCCCTT AGCCATCCGC GAACGGATGC
CCANNNNNNA CCGCGAGGAG GTGGACCCTG NNNN-3' (SEQ ID NO:5),
where N is A, C, G or T; N₄₄₋₄₉ is the sequence of the P1 stem and N₇₁₋₇₄ is the
sequence of the blocker as illustrated in a preferred embodiment as set forth in
5 on SEQ ID NO:1); and, ii) the sense primer (T7-5'Rz-up; 5'-TTAATACGAC
TCACTATAGG GCCAGCTAGT TT(N)_{7-20-BS}(N)_{4-BL} CAGGGTCCAC C-3': SEQ
ID NO:6) that permitted the incorporation of the T7 RNA promoter, and where
(N)_{7-20-BS} represents the biosensor (BS) sequence of 7 to 20 ribonucleotides in
length and where (N)_{4-BL} represents the blocker sequence (BL) of 4
10 ribonucleotides in length. The same strategy using two oligodeoxynucleotides
was used to build the different versions of the ribozyme (i.e. the variants in the
Biosensor (Fig. 10 and 11); the Blocker (Fig. 12); the Stabilizer (Fig. 14); and,
the SOFA- δ Rz-DN- and SOFA- δ Rz-DB ribozymes (Fig. 17)). All sequence
variants are depicted in the corresponding figure. The amplification method has
15 been described previously (Bergeron, L.J., & Perreault, J.P. *Nucleic Acids Res.*
30, 4682-4691, 2002). The PCR products were purified by phenol:chloroform
extraction, precipitated with ethanol and dissolved in water. *In vitro*
transcriptions and purifications of the ribozymes were then performed as
described below. A similar strategy was used to build the different versions of δ
20 ribozyme.

The same strategy was used to construct substrates a to h of Fig. 10.
Table 1 describes the sequences of the antisense primer.

TABLE 1
Oligodeoxynucleotide sequences of the a to h substrates

a	5-AAAGT <u>GAGACAAGAA</u> TGTGAAACCAC / AAGAG CCCTATAGTGAGTCGTATTAA-3' (SEQ ID NO:7)
b	5-AAAGT <u>AGACTGAGAT</u> ATGTGAAACCAC / AAGAG TGTA CT CCCTATAGTGAGTCGTATTAA-3' (SEQ ID NO:8)
c	5-AAAGT <u>GTTCA</u> GCACTATGTGAAACCAC / AAGAG TGTA CT GTCCCTATAGTGAGTCGTATTAA-3' (SEQ ID NO:9)
d	5-AAAGT <u>AGGATACGGG</u> ATGTGAAACCAC / AAGAG TGTA CT GTAA CC CTATAGTGAGTCGTATTAA-3' (SEQ ID NO:10)
e	5-AAAGT <u>AGTCTGGAT</u> CATGTGAAACCAC / AAGAG TGTA CT GTAA CT CCCTATAGTGAGTCGTATTAA-3' (SEQ ID NO:11)
f	5-AAAGT <u>GGCATAATCA</u> ATGTGAAACCAC / AAGAG TGTA CT GTAA CT CCCCCTATAGTGAGTCGTATTAA-3' (SEQ ID NO:12)
g	5-AAAGT <u>AAGTTGGCGA</u> ATGTGAAACCAC / AAGAG TGTA CT GTAA CT TCAACCCTATAGTGAGTCGTATTAA-3' (SEQ ID NO:13)
h	5-AAAGT <u>GTA</u> CTCATGCATGTGAAACCAC / AAGAG TGTA CT GTAA CT TCAATGCCCTATAGTGAGTCGTATTAA-3' (SEQ ID NO:14)
(/) indicates the P1 cleavage site. The biosensor binding sequence is underlined.	

For the substrates with seven different spacer lengths, the antisense
 5 primers were: 5'-AAAGTGAGACAAGAA-(A)_{0-6nt}-
 (AAACCAC)₇AAAAAACCCTATAGTGAGT CGTATTAA-3' (SEQ ID NO:15),
 where the T7 promoter sequence is underlined.

For the substrates with the spacers of different lengths; but possessing a
 unique cleavage site, the antisense primers were: 5'-
 10 AAAGTGAGACAAGAA(AAAAC)_{SP}-(ACCAACA)_X(AAACCAC)_Y(ACCAACA)_Z-
 AAAAAACCCTATAGTGAGTCGTATTAA-3'(SEQ ID NO:16) (where SP is for
 spacer, the number of X and Z units varied as desired; the unit Y gives the

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cleavable P1 sequence; and, the T7 promoter sequence is underlined). In these cases, the spacer was always 5'-AAAAC-3', except for the substrate of 5 nt that included the sequence 5'-AAAAA-3'.

For the *in vivo* experiments, the open reading frame of the HBV C gene
5 was amplified from pCH9T/3091 (Nassal, M. J. Virol. 66, 4107-4116, 1992) using forward primer (5'-TATCTAAAGC TAGCTTCATG TCCTACTGTT CAAGCCTCC-3', SEQ ID NO:17) and reverse primer (5'-TAGTGAAACT CGAGAATAAA GCCCAGTAAA GTTCCCA-3', SEQ ID NO:18). The DNA product was cloned in the multiple cloning site of the pIND™ vector (Invitrogen)
10 at the Nhe1 and Xho1 restriction sites. The strategy for the design of the vector expressing the ribozymes included several steps: 1) Firstly, the vector pcDNA3™ (Invitrogen) was digested at the Hind III and Xho I sites removing a portion of the multiple cloning sites region; 2) Secondly, a cassette was synthesized using two overlapping oligodeoxynucleotides (5'-AGCTTGGTAC
15 CGAGTCCGGA TATCAATAAA ATGC-3', SEQ ID NO:19 and 5'-TCGAGCATTT TATTGATATC CGGACTCGGT ACCA-3', SEQ ID NO:20 allowing introduction of Knp I and EcoR V restriction sites followed by a poly(A) signal sequence. These modifications of the vector permitted the production of ribozymes with a 3'-end poly(A) tail allowing their localization in the cytoplasm. This modified
20 pcDNA3™ version was named pmδRz for "plasmid messenger δ ribozyme". 3) Thirdly, the product of amplification for the synthesis of ribozymes described above, was used to perform a new PCR amplification using as forward primer 5'-ATCCATCGGG TACCGGGCCA GTTAGTTT-3' (SEQ ID NO:21), and reverse primer 5'-CCAGCTAGAA AGGGTCCCTT AGCCATCCGC G-3' (SEQ

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ID NO:22). This nested PCR allowed removal of the T7 RNA promoter sequence and introduction of a 5'-end Kpn I site and a 3'-end blunt sequence; 4) The resulting PCR products have been cloned in the Kpn I and EcoR V linearized pcDNA3™ modified version (i.e. prepared in step 1 and 2). All sequences were confirmed by DNA sequencing.

RNA synthesis

Both ribozymes and RNA substrates were synthesized by run-off transcription from PCR products, *Hind*III linearized plasmid pHBV-1190 and *Xba*I linearized plasmid pHCVa templates. Run-off transcriptions were performed in the presence of purified T7 RNA polymerase (10 µg), RNAGuard™ (32 units, Amersham Biosciences), pyrophosphatase (0.01 units, Roche Diagnostics) and linearized plasmid DNA in a buffer containing 80 mM HEPES-KOH, pH 7.5, 24 mM MgCl₂, 2 mM spermidine, 40 mM DTT, 5 mM of each NTP and with or without 50 µCi [α -³²P]UTP (New England Nuclear) in a final volume of 100 µL at 37°C for 3 hrs. Upon completion, the reaction mixtures were treated with DNase RQ1™ (Amersham Biosciences) at 37°C for 20 min, purified by phenol:chloroform extraction, and precipitated with ethanol. The viral RNA products and ribozymes were fractionated by denaturing 5% and 8%, respectively, polyacrylamide gel electrophoresis (PAGE; 19:1 ratio of acrylamide to bisacrylamide) in buffer containing 45 mM Tris borate, pH 7.5, 7 M urea and 1 mM EDTA. The reaction products were visualized by either UV shadowing or autoradiography. The bands corresponding to the correct sizes of the ribozymes and the viral RNAs were cut out and eluted overnight at room temperature in a solution containing 0.5 M ammonium acetate and 0.1 % SDS.

The transcripts were desalted on Sephadex G-25™ (Amersham Biosciences) spun-columns, and were then precipitated, dissolved and quantified either by absorbance at 260 nm or ³²P scintillation counting.

Labelling of RNA substrates

5 First, RNA substrate (20 pmoles) was dephosphorylated using 0.2 units of calf intestinal alkaline phosphatase according to the manufacturer's recommendations (Roche Diagnostics). The reactions were purified by extracting with phenol:chloroform and precipitated with ethanol. Subsequently, the RNAs (10 pmoles) were 5'-end labelled in a mixture containing 10 µCi
10 [γ -³²P] ATP (3000 mCi/mmol; New England Nuclear) and 12 units of T4 polynucleotide kinase following the manufacturer's protocol (United States Biochemicals). The end-labelled RNAs were purified using denaturing PAGE, and the relevant bands excised from the gel, then eluted, precipitated, and dissolved in water.

15 Ribozyme cleavage assays

Except when indicated, all reactions were performed under single turnover conditions ($[Rz] > [S]$, where $[Rz]$ is the ribozyme concentration and $[S]$ is the substrate concentration) using 1 µM ribozyme and trace amounts of either internally ³²P-labelled or ³²P 5'-end labelled 1190 nt HBV, 1422 nt HCV or
20 shorter RNA substrates at 37°C in a final volume of 10 µL containing 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂. For the multiple turnover reactions, the assays were performed at 50°C with an excess of substrate over the ribozyme (15 µM vs 1 µM, respectively). After 3 hrs of incubation, the reactions were

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stopped by adding the loading buffer (5 μ L of 97% formamide, 10 mM EDTA, pH 8.0), loaded on a 6% Polyacrylamide gel and analyzed with a radioanalytic scanner (PhosphorImager™, Molecular Dynamics). For the time course experiments, aliquots (0.8 μ L) were removed at various times, up to 3 hrs, and
5 were quenched by the addition of 5 μ L of ice-cold formamide dye buffer. Cleavage reactions for the mechanism analysis were carried out either with or without 5 μ M of a facilitator (FCO, 5'-AAAGTGAGAC AAGAA-3', SEQ ID NO:23), biosensor stem (BSO, 5'-TTCTTGTCTC ACTTT-3', SEQ ID NO:24) and an unrelated (UNO, 5'-CCCAATACCA CATCA-3', SEQ ID NO:25)
10 oligodeoxynucleotide. Cleavage assays with the pools of mixed substrates were performed with trace amounts of radiolabelled substrates (50 000 cpm), non-labelled RNA substrate (2 μ M) and SOFA-ribozymes (500 nM), except for the original ribozyme (WT, 2 μ M). The reactions were incubated for 2 hours, analyzed on denaturing 10% Polyacrylamide gels, and revealed by
15 PhosphorImager™.

RNase H hydrolysis of SOFA- δ Rz-303

Trace amounts of 5' end labelled SOFA- δ Rz-303 (~10 000 c.p.m; < 0.1 pmol) in the presence of 50 pmol of either the unlabeled small substrate (44 nt) or yeast tRNA as carrier (Roche Diagnostic) were preincubated in a volume of 8
20 μ L containing 25 mM Tris-HCl pH 7.5, 25 mM KCl, 12 mM MgCl₂, 0.13 mM EDTA and 0.13 mM DTT at 25°C for 10 min. Then, oligodeoxynucleotides (L3': 5'-GCGAGGA-3'; P4': 5'-CCATCCG-3'; BS': 5'-TGTCTCA-3'; BL': 5'-TGAAACT-3' and ST': 5'-CAGCTAG-3') 7 nt in size (10 pmol; 1 μ L) were separately added to the samples before pre-incubating for another 10 min. Finally, 2 units of

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Escherichia coli RNase H (Ambion; 1 μ L) were added to the mixtures and the samples incubated at 37°C for 10 min. The reactions were quenched by adding 5 μ L of cooled stop solution (97% formamide, 0.025% xylene cyanol and 0.025% bromophenol blue), the samples fractionated on denaturing 8% PAGE gels and the gels analyzed with a radioanalytic scanner (StormTM).

Cell culture and DNA transfections

HEK 293 EcR cells (Human Embryonic Kidney) were grown in Dulbecco's modified Eagle's medium (DMEMTM) (Sigma) supplemented with 10% fetal bovine serum (Wisent) and 0.4 mg/ml of zeocine (Invitrogen) at 37°C in 5 % CO₂. The cells were transfected using LipofectamineTM, as per the manufacturer's instructions (Invitrogen).

Northern Blot Hybridization

Total RNA from HEK 293 EcR was extracted with Tri-Reagent (Bioshop Canada Inc, Burlington, Ontario, Canada). Northern blot analyses of total RNA (10 μ g) extracted from HEK 293 EcR cells, were performed as described previously (D'Anjou, F., et al., J. Biol. Chem. 279, 14232-14239, 2004). The probes were synthesized as followed. For the HBV gene C probe, aliquot of the PCR product obtained previously (see DNA construct section) was cloned in the Xba I and Xho I sites of the pBlueScriptTM (SK) vector (Stratagene). The resulting Sac I linearized vector was transcribed *in vitro* using T7 RNA polymerase in the presence of [α -32P]UTP. The β -actin RNA probe was synthesized using the Strip-EZTM RNA T7/T3 kit (Ambion) according to the manufacturer's conditions. All hybridizations were carried out for 16-18 h at

65°C. The membranes were exposed on PhosphorImager™ screen for 2-24 h. The densitometry analysis was carried out on ImageQuant™ software.

RESULTS

The SOFA-ribozyme of the present invention was tested using two
5 accessible sites of the hepatitis B virus (HBV) RNA that have been previously
selected for ribozyme cleavage (δ Rz-303 and δ Rz-513)(Bergeron, L.J., &
Perreault, J.P. *Nucleic Acids Res.* **30**, 4682-4691, 2002). These ribozymes
inefficiently cleaved an HBV-derived RNA of 1190 nucleotides (nt) (~15 %; Fig.
3). Corresponding SOFA-ribozymes (SOFA⁺) possessing a biosensor that
10 basepairs five nucleotides downstream of the P1 stem binding site demonstrate
a drastically improved level of cleavage activity at both sites (~75%). Ribozymes
bearing a biosensor domain unrelated to the target sequence remain locked in
an inactive conformation (SOFA⁻). The latter did not exhibit significant levels of
cleavage activity even though the nucleotide sequence of the *delta* ribozyme
15 portion is identical. This shows a greater "safety lock" capacity provided by the
blocker domain, thereby diminishing the risk of non-specific cleavage. In Fig. 3,
the length of the bands, in nucleotides, is shown adjacent to the gel. The control
(-) was performed in the absence of ribozyme, while SOFA⁺ and SOFA⁻
indicates SOFA harbouring either the appropriate or inappropriate biosensor
20 sequence, respectively.

Time course experiments reinforced the conclusion that appending a
specific SOFA significantly contributes to enhancing the cleavage activity (Fig.
4). Moreover, similar conclusions were reached for several other SOFA-

ribozyme constructions cleaving either HBV or hepatitis C virus (HCV) (see Table 2). Variation in the levels of cleavage activity might be due to several features, including the differences in binding efficiencies and the rate constants of the SOFA structural transitions (*on/off* conformations).

5

Table 2
Enzymatic activity of various SOFA-ribozyme constructions

Target	Position	Open reading frame	% of cleavage	
			WT	SOFA
HBV	167	C gene	3,6	4,0
	279	C gene	18,5	57,9
	303	C gene	26,1	71,6
	398	C gene	-----	79,0
	513	C and P gene	16,0	54,7
	993	S and P gene	-----	57,4
HCV	224	IRES	1,3	13,4
	302	IRES	3,3	29,4

Cleavage activity was investigated using SOFA⁺-Rz-303 possessing biosensor sequences of various lengths. Under single turnover conditions, the cleavage levels increased in proportion to the size of the biosensor (Fig. 5). The longer the base-paired segment (BS-7 < BS-10 < BS-12 < BS-15 < BS-20), the better the binding of the ribozyme to the substrate and the higher level of cleavage activity. Kinetic analyses permitted the determination of second order rate constants (k_{cat}/K_M) where it was observed that those for the SOFA⁺-ribozymes were up to an order of magnitude higher than that of the original ribozyme (WT). Under multiple turnover conditions, a smaller HBV-derived substrate was used since the length of the latter affects the turnover rate of the

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ribozyme. In this case, the level of cleavage increases in proportion to the length of the biosensor, up to 10 nucleotides, at which point it decreased with increasing length (Fig. 6). Elongation of the biosensor stimulates the cleavage activity up to the point where product release becomes rate limiting.

5 Astonishingly, in this specific experiment the SOFA⁺- δ Rz-303-BS-10 performed four turnovers, while the original ribozyme only completed one. More importantly, the SOFA-ribozyme meets the classical criteria of an enzyme (e.g. it exhibits turnovers). Since no independent effector is required, this is not an allosteric enzyme. In Fig. 6, the XC indicates the position of the xylene cyanol.

10 The length of the bands in nucleotides is shown adjacent to the gel. The control (-) was performed in the absence of ribozyme.

The mechanism of action of both the biosensor and blocker sequences was investigated using an oligodeoxyribonucleotide competition approach coupled with mutated ribozymes (Figs. 7A and 7B). In Fig. 7A, the roman

15 numerals identify the steps of the mechanism. Dashed lines identify oligodeoxyribonucleotide binding to either the substrate (i.e. FCO acting as facilitator) or the biosensor (BSO). The addition of an oligodeoxyribonucleotide with the same sequence as the biosensor domain (FCO) slightly increased the level of cleavage of the original δ Rz-303. This oligodeoxyribonucleotide acts as

20 a facilitator that renders the binding site more accessible to the catalytic region of the ribozyme. In contrast, the presence of the FCO does not alter the level of cleavage of SOFA⁺- δ Rz-303 after an incubation of 3 hours, although it takes more time to reach this cleavage level. One possible explanation is that the binding of both the biosensor and the P1 sequence favourably competes with

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the FCO for the substrate. When the experiment is repeated using an oligodeoxyribonucleotide complementary to the biosensor sequence (BSO), the cleavage activity of the SOFA⁺-ribozyme is drastically decreased. Conversely, the presence of an oligodeoxyribonucleotide having an unrelated sequence
5 (UNO) does not modify the cleavage level, indicating that the biosensor domain is the driving force in the process. Finally, the contribution of the blocker domain was assessed using SOFA⁻- δ Rz-303. This *off*-version which lacks the adequate biosensor, but possesses the appropriate P1 stem, barely demonstrated a detectable level of cleavage. In contrast, a mutant lacking the blocker sequence
10 exhibited a higher cleavage activity than did the SOFA⁻-ribozyme. Hence, the blocker plays its role by preventing the formation of the P1 stem in the absence of the appropriate biosensor. This requirement increases the specificity of the SOFA because it can only be activated by the desired substrate.

The specificity of a ribozyme can commonly be defined by the ability to
15 discriminate between two or more similar RNA substrates. In order to illustrate the gain in terms of substrate specificity, two distinct experiments were performed. First, ten substrates were designed (see Table 3 below), each possessing an identical P1 binding sequence coupled to a distinct binding sequence for the biosensor. The substrates were successively extended at their
20 5' extremity by at least two nucleotides in order to provide them with assorted electrophoretic mobilities (Fig. 8A).

Table 3
Substrates

Substrates (SEQ ID NO)	Substrates sequences				
	5'	P1	Spacer	BS	3'
A (SEQ ID NO:26)	GGGCUCUU	GUGGUUU	CACAU	UUCUUGUCUC	ACUUU
B (SEQ ID NO:27)	GGGUACACUCUU	GUGGUUU	CACAU	CAGGCACCUC	ACUUU
C (SEQ ID NO:28)	GGGAGUACACUCUU	GUGGUUU	CACAU	AUCUCAGUCU	ACUUU
D (SEQ ID NO:29)	GGGACAGUACACUCUU	GUGGUUU	CACAU	AGUGCUGAAC	ACUUU
E (SEQ ID NO:30)	GGGUUACAGUACACUCUU	GUGGUUU	CACAU	CCCGUAUCCU	ACUUU
F (SEQ ID NO:31)	GGGAGUUACAGUACACUCUU	GUGGUUU	CACAU	GAUCCAGACU	ACUUU
G (SEQ ID NO:32)	GGGGAAGUUACAGUACACUCUU	GUGGUUU	CACAU	UGAUUAUGCC	ACUUU
H (SEQ ID NO:33)	GGGUUGAAGUUACAGUACACUCUU	GUGGUUU	CACAU	UCGCCAACUU	ACUUU
I (SEQ ID NO:34)	GGGCAUUGAAGUUACAGUACACUCUU	GUGGUUU	CACAU	GCAUGAGUAC	ACUUU
J (SEQ ID NO:35)	GGGUGCAUUGAAGUUACAGUACACUCUU	GUGGUUU	CACAU	CUGUGCUGCA	ACUUU

When all of the substrates were incubated together with a given SOFA⁺-
5 ribozyme, only the substrate harbouring the relevant requirements, in term of
sequence recognition by the biosensor of that ribozyme, was cleaved. Lower
cleavage levels for the substrates **B** and **J** were indicative of the influence of the

biosensor sequence identity. This experiment reveals that a ribozyme, activated by the proper substrate, did not cleave other substrates via a *trans*-cleavage mechanism. Conversely, the original ribozyme did not make this discrimination (lane WT) and all substrates were cleaved, although at different levels. In a
5 second demonstration, the inventors attempted to selectively cleave sites of long RNA molecules that included an identical P1 binding sequence but different biosensor sequences. A sequence of 7 nt long was retrieved twice in the HBV fragment (i.e. cleavage at positions 398 and 993; Figs. 8B and 8E), demonstrating the possibility of having multiple cleavage sites using the 7 nt
10 requirement of a wild type ribozyme. In addition, the classical HDV ribozyme did not allow the detection of cleavage at either of these sites, most likely because they were embedded in complex structures. In contrast, SOFA-ribozymes exhibited an efficient and specific cleavage at these sites (i.e. without any interference between the sites). This corroborates the power of the SOFA
15 module, and points out that it enables the cleavage of a substrate uncleavable by the original ribozyme. Similar results were acquired when targeting a repeated sequence (i.e. cleavage site at positions 224 and 302) within the highly structured Internal Ribosome Entry Site (IRES) of the hepatitis C virus of HCV; Figs. 8C and 8F). Consequently, no *cis*-cleavage was observed in these
20 studies. Clearly, these experiments establish the high substrate specificity of the SOFA module of the present invention, in addition to illustrating its character as a facilitator (i.e. unwinding the neighbouring structure of the target site). The accessibility of target sites became a less important hurdle than it was for classical ribozymes (D'Anjou, F., et al., *J. Biol. Chem.*, 279, 14232-14239,
25 2004). In Figs. 8A and 8D, there is reported cleavage assays of a pool of ten 5'-

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end-labelled substrates (a to j) by either specific SOFA-ribozymes (named A to J) or the original ribozyme (WT). All the ribozymes and substrates have a similar P1 stem sequence (P1), but differ in the biosensor sequences (BS). The length of each substrate is indicated in nucleotides. BPB and XC in Fig. 8D indicate the positions of bromophenol blue and xylene cyanol, respectively. The control (-) was performed in the absence of ribozyme. In Figs. 8B and 8E, there are reported cleavage assays of the HBV-derived target by SOFA-ribozymes cleaving at either position 398 or 993. In Figs. 8C and 8F, there are reported cleavage assays of a 1422-nt HCV-derived target by SOFA-ribozymes cleaving at either position 224 or 302 of the IRES. For Figs. 8B, 8E, 8C and 8F, the sequence of the P1 stem is identical at each site, but the biosensor sequences are different.

The specific sequences of the various studied targeting sites derived from HBV and HCV viruses are illustrated in Fig. 9 (SEQ ID NO:36 to 41). The cleavage sites are indicated with arrows.

Specificity conferred by the biosensor sequence

In order to gain more knowledge from the SOFA module, we were interested in establishing the substrate specificity for ribozyme cleavage conferred by the biosensor sequence. Several experiments described above revealed the crucial role that the biosensor sequence must play in order to insure great accuracy in terms of the substrate specificity for the ribozyme cleavage. More specifically, it has been shown that a ribozyme, when activated by the proper substrate, does not cleave other substrates by either the *cis*- or

trans-cleavage mechanisms. For example, in one experiment a ribozyme was incubated in the presence of ten different small substrates possessing identical P1 binding sequences coupled to completely different biosensor binding sequences (Fig. 8A and D). This experiment led to the conclusion that each of the ten ribozymes only cut efficiently when the biosensor perfectly bound to the target RNA. However, it did not permit investigation of how the biosensor sequence identity influenced the substrate specificity. In order to address this issue we performed two distinct experiments.

Initially, the cleavage activities of the eight most active SOFA-ribozymes from the collection described above were determined for each substrate alone, rather than within a pool. Both the biosensor sequences of the ribozymes and the substrate sequences are shown in Fig. 10A (SEQ ID NO:42 to 57). The substrates were arbitrarily designated a to h, while a ribozyme perfectly complementary (i.e. one with the appropriate biosensor, SOFA⁺) to a given substrate received the corresponding superscript letter, i.e. A to H (e.g. RzS = Aa). These experiments were performed under single turnover conditions ([Rz]>[S]). Aliquots were removed at different intervals, fractionated on denaturing 10% polyacrylamide gels (PAGE) and analyzed by radioanalytic scanning. A typical gel is shown in Fig. 10B and shows the kinetics for the couple Dd. The cleavage rate constant (k_{obs}) for at least two independent assays was estimated for each possible couple. The reaction time course for each substrate with ribozyme D is illustrated in Fig. 10C. Only the perfectly matched couple Dd exhibited an active cleavage. The k_{obs} average values for each RzS couple tested (64 couples) are reported as a histogram (Fig. 10D). This large data set prompted several observations. First, only the eight couples

that had a perfect match between the biosensor and substrate sequences (located on the diagonal) had high k_{obs} values. These k_{obs} values varied between 0.056 to 0.69 min^{-1} (i.e. Cc and Ee, respectively). This difference of 12-fold in the k_{obs} shows that the identity of the biosensor sequence significantly
5 influenced the cleavage activity.

Secondly, most of the imperfect couples, in which the number of mismatches varied between two and eight (the GU wobble was considered as base pair (bp)), exhibited cleavage activities characterized by significantly lower k_{obs} . In several cases the k_{obs} values for the cleavage of a mismatched substrate
10 were three or more orders of magnitude smaller than that of their perfectly matched counterparts (e.g. Ac, Dg, and Hd). For example, ribozyme H cleaves substrate d with a rate constant 15 000 times smaller than it does substrate h. However, in most of the cases, the rate constants of imperfect couples were 25 to 250 fold lower. Thus, ribozyme F cleaved the imperfect substrates with k_{obs}
15 varying from 25 to 47 fold smaller than that of the perfect substrate, while ribozyme E cleaved them with k_{obs} values ranging between 77 to 291 fold less than that of the perfect substrate (with the exception of the Ed couple that possessed a k_{obs} 5010 times smaller than that of the ideal Ee pair). More generally, we observed that the catalytic parameters correlate directly with
20 substrate specificity (i.e. the more active the ribozyme, the better its substrate specificity seemed to be). Additional ribozymes with a different biosensor sequence (i.e. one with more than three mutations) also led to the same conclusion; namely that they efficiently cleaved their desired substrate (that with the sequence complementary to the biosensor), but not other unrelated

substrates. Together, these results demonstrate the potential of the biosensor to improve the substrate specificity of a ribozyme.

This first experiment confirms that a ribozyme cleaves its perfectly complementary substrate with a relatively high rate constant value, but that it is drastically less efficient for non-perfect couples (i.e. those including several mismatches). In order to obtain a more precise picture of the situation, a second experiment involving SOFA- δ Rz-303 sequence variants with less potential for forming mismatches was performed. Twenty-three mutated ribozymes including 1 to 4 randomly distributed substitutions within the biosensor sequences were synthesized (Fig. 11A, SEQ ID NO:58 to 81). A residue of the biosensor was substituted for by the same base found at the corresponding position within the substrate, thereby producing a mismatch. The cleavage activity of each mutated ribozyme was assessed, and the rate constant (k_{obs}) determined. The k_{obs} are reported individually in panel A of Fig. 11, while panel B illustrates the variation of the k_{obs} average as a function of the number of mutations. Clearly, the decrease in the cleavage activity is directly related to the number of mutations (Fig. 11B). While the presence of a single mismatch reduced the k_{obs} values from 4 to 15 fold, the presence of 4 mutations yielded k_{obs} values 18 to 106 fold smaller. The position of the mutation within the biosensor appeared to have only a small effect on the cleavage observed. However, a single mutation in the middle of the biosensor stem reduced the cleavage activity slightly more than one located near the ends (see Fig. 11A). This is probably due to the fact that a mismatch in the middle of the stem may interrupt the stacking. According to these data, the presence of only one mutation in the biosensor appears to be sufficient to significantly affect the cleavage activity. Two different mutants were

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produced for the positions 2, 6, 9 and 6-9, and the decrease in the cleavage activity was found to be similar regardless of the nature of the mutation (see Fig. 11A). In addition, we also observed that the influence of a mutation in the biosensor was more important when targeting a long HBV-derived transcript.

5 This suggests that SOFA- δ Rz efficiently discriminate their substrate. The second order rate constants (k_{cat}/K_M) of both the single and double mutants, SOFA- δ Rz-303(A6U) and (A6U)(A9U), were shown to be 25-fold lower than that of the original version (stars in Fig. 11A; see above). Determination of the kinetic parameters of other single or double mutants also led to the same

10 conclusion (i.e. the k_{cat}/K_M values of the mutants are at least one order of magnitude lower than that of the original SOFA- δ Rz-303, stars in Fig. 11A). These differences were also due to a lower k_{cat} and a higher K_M , in agreement with the idea that fewer base pairs are involved in the recognition between the ribozyme and the substrate.

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Characterization of the blocker sequence

In the absence of the appropriate target RNA substrate, the SOFA-ribozyme adopts an inactive conformation, the *off* conformation. According to the SOFA design, this state is due to the 4 nucleotides blocker sequence

20 binding the P1 region of the ribozyme, thereby preventing the binding of non-specific substrates (see Fig. 2). Consequently, the longer the blocker sequence, the better the "safety lock" effect. In order to verify this hypothesis, and to establish the importance of the blocker sequence for the "safety lock" concept, several SOFA-ribozymes with mutated blocker sequences were synthesized

25 and their cleavage activities assessed by targeting the HBV-derived transcripts

of 1190 nucleotides. Since no mutation was required within the substrate, the longer transcript appeared to be more suitable for characterization because it is more relevant to a natural target. Different blocker lengths (0 to 5 nucleotides) were used in order to find the largest stem that did not inhibit cleavage of the appropriate substrate (Fig. 12A). A typical autoradiogram of a PAGE gel is illustrated in Fig. 12B. In the absence of the blocker sequence, SOFA- δ Rz-303 was very active (i.e. BL-0, 81% cleavage). The same level of cleavage was detected in the presence of a 2 nucleotides blocker sequence (i.e. BL-2, 79% cleavage), indicating that two bases were insufficient to allow the formation of a stable stem between the blocker and the ribozyme's P1 strand. A SOFA-ribozyme with a 4 nucleotides blocker sequence cleaved the substrate relatively efficiently, although at a reduced level as compared to the previous assay (i.e. BL-4 71%). Elongation of the blocker sequence by one more nucleotide significantly reduced the cleavage exhibited (i.e. BL-5 40% of cleavage). In this case, it seems that the ribozyme remained locked in the *off* conformation, indicating that formation of the intramolecular stem between the P1 region of the ribozyme and the blocker sequence seems to be favoured over hybridization between the ribozyme and the substrate. Thus, a blocker sequence of 4 nucleotides appears to be the optimal size to lock the ribozyme while still allowing it to unlock in the presence of the desired substrate. Time-course experiments of these four ribozymes confirmed that a blocker sequence of 4 nucleotides is suitable for establishing a balance between the *off/on* conformations, that is one that blocks, but not too much (Fig. 12C).

Blockers of 6 nucleotides or more were also tested. In addition to blocking too much of the ribozyme in its inactive conformation (i.e. almost

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irreversible), we also observed ribozymes that self-cleaved the sequence adjacent to the blocker sequence (i.e. within the biosensor), an unacceptable phenomena.

The sequence of the blocker segment might also modulate the level of inhibition. We observed that if a mutated blocker cannot form a stem with the P1 strand, then no inhibition is observed. In contrast, previous experiments have shown that SOFA⁺- δ Rzs with different target sites on HBV-derived transcripts were all inactive (see previously). These ribozymes possessed the appropriate P1 strands and complementary blocker sequences, while their biosensor sequences could not bind the substrates. The inactivity of these SOFA⁺- δ Rzs confirmed that the blocker sequence plays its role by inhibiting the catalytic activity in the absence of the appropriate biosensor sequence. In all cases, the SOFA⁺- δ Rzs possessing a biosensor sequence capable of binding the substrate efficiently cleaved their substrates.

Spacing between the P1 stem and the biosensor binding domain

A SOFA-ribozyme recognizes its substrate through two independent domains. Initially, the biosensor sequence binds its complementary sequence on the substrate, and, subsequently, the P1 stem is formed between the ribozyme and the substrate. In all experiments reported so far, the two binding domains were separated by 5 nucleotides simply to avoid the chance that the proximity and stacking of the P1 and biosensor would affect the release of the product. However, there was no scientific rationale supporting this spacing of 5 nucleotides. In order to investigate this parameter seven model substrates possessing seven head-to-tail repetitions of the P1 stem domain (P1_N) followed

by the SOFA- δ Rz-303 biosensor sequence were synthesized (see Fig. 13A). The substrates differed by possessing a distance of 0 to 6 nucleotides between the domain bound by the biosensor and the first adjacent P1 binding sequence. In this way we created the equivalent of 49 different substrates that included

5 different spacer lengths. The ribozyme should bind its complementary sequence at the 3' end of the substrate via its biosensor, and should subsequently find a P1 sequence at an ideal distance. The cleavage experiments were performed during a short period of time (5 min) so as to permit only the unique cleavage reaction of 5' end labelled substrates to occur. The substrates used in this

10 experiment exhibited different electrophoretic mobilities depending on their sizes, which differed by one nucleotide. The 5' radiolabelled products of all cleavages made with the same P1 sequence migrated similarly on the gel because the one base difference was located within the non-radioactive 3' product (Fig. 13B). We observed that all substrates were preferentially cleaved

15 at the first or second sites near to the biosensor sequence (i.e. P1₁ and P1₂). With the exception of the substrate with no spacing between the P1 and biosensor domains, we observed that the higher level of cleavage occurred at the first P1 site (P1₁). In order to facilitate the interpretation of this data, we calculated the relative percentage of cleavage for all substrates. They are

20 shown as a function of the spacer length (Fig. 13C). There is an increase in the percentage of cleavage as one progress from no spacer to an optimal length of 3 nucleotides. This is followed by a decrease up to a spacer of 21 nucleotides, at which point the relative percentages of cleavage remains constantly low regardless of the length of these sequences. The decrease occurs mainly

25 stepwise for substrates cleaved at their P1₂, P1₃, and P1₄ sequences, with a

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preference for the substrates with the smaller spacers (see dashed lines, Fig. 13C). This experiment shows that it is preferable to have at least a one nucleotide space between the biosensor and the P1 region, and that the optimal length is found between 1 and 5 nucleotides. The need to have at least a
5 minimal spacer was confirmed by observing that a completely different SOFA- δ Rz barely cleaved its substrate if there is no spacer between the substrate's P1 domain and the biosensor.

We subsequently confirmed these results using different substrates that harbour spacers of different lengths and a single cleavage site, like the normal
10 SOFA- δ Rz does. Four substrates were designed based on the initial results obtained with the seven consecutive P1 stem domains. Each of these substrates contained only one P1 sequence, located in position P1₁, P1₃, P1₅ or P1₇ (i.e. 5'-GUGGUUU-3'). The other sequences were replaced by another that cannot be bound by the P1 strand of the ribozyme (i.e. 5'-UGUUGGU-3'). In this
15 way, the spacer sequences were extended to 5, 19, 33 and 47 nucleotides, respectively. All substrates were cleaved at different levels (see Fig. 13D, inset). The relative percentages of cleavage were used to analyze the effect of the spacer length. (Fig. 13D). We observed that the shorter a spacer is, the better the cleavage activity of the ribozyme. However, the difference between the
20 shorter and longer spacers, in terms of cleavage activity, is not as significant as in the above experiment. In the present case, there is no competition between several sites, a condition that should enhance the level of catalytic activity regardless of the position of the cleavage site. These data confirmed that a minimal spacer (1 to 5 nucleotides) is better for efficient activity with SOFA- δ Rz.

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The sequence of the stabilizer stem does not influence the ribozyme cleavage. The stabilizer brings both the 5' and 3' ends into a common terminal stem. This domain has been included in the SOFA module due to previous observations revealing that the terminal P2 stem of the original δ Rz provides tremendous stability to this RNA species (Lévesque et al., *RNA* **8**, 464-477, 2002). It was also shown above that the presence of the stabilizer within the SOFA-module increases the stability of SOFA- δ Rz-303. Here, we address the influence of the stabilizer domain, which does not have an active role in the SOFA mechanism. Both the SOFA⁺- and SOFA⁻- δ Rz-303 versions, with or without stabilizer sequences, were constructed and used to define the influence of this domain on the cleavage activity (Fig. 14A). As we first thought, the two SOFA⁺- δ Rz-303 versions exhibited the same level of cleavage activity regardless of the presence (lane 3) or absence (lane 5) of the stabilizer sequences, while their SOFA⁻ counterparts (i.e. those without the appropriate biosensor sequence) were inactive (lanes 4 and 6). These observations confirmed that the stabilizer sequences did not interfere with the cleavage activity of the SOFA-ribozyme.

Subsequently, the stabilizer was mutated to five different base pairs (see Fig. 14B, SOFA- δ Rz-303-ST2 as compared to -ST1) and used in the cleavage assay. As expected, both versions of SOFA-ribozyme exhibited virtually identical levels of cleavage. Similar results were observed if the mutation allowed the formation of only 2 bp within the stabilizer (Fig. 14B, SOFA- δ Rz-303-ST3 as compared to -ST1). These results are in good agreement with the hypothesis that the identity of the stabilizer sequence does not affect the SOFA-module action.

Surprisingly, another mutant (SOFA- δ Rz-303-ST4) exhibited a drastic decrease in cleavage activity, a result that contradicts all of the data previously presented. We analyzed the sequence of this ribozyme in detail and realized that the 5'-strand of the stabilizer (5'-CCUCGAAC-3') was complementary to a stretch of sequence located within the P4 stem-loop (5'-GUUCGCGG-3'). This observation suggests that this stabilizer could interact with the P4 stem-loop of the ribozyme and thereby influence the structure of the ribozyme itself.

Structural analysis of SOFA- δ Rz-303

10 In order to probe both the *off* and *on* conformations of SOFA- δ Rz-303, we used an approach based on an oligodeoxynucleotide hybridization assay in order to distinguish between single and double stranded domains. The *off* and *on* conformations were probed in both the absence and the presence (in excess) of the 44 nucleotide model substrate (SEQ ID NO:82). With the goal of preventing cleavage, we used a SOFA-ribozyme in which the cytosine in position 76 is replaced by an adenosine (Fig. 15A, SOFA⁺- δ RzC76A-303, SEQ ID NO:83). The SOFA-ribozyme that possesses this mutation has the same binding ability as the original, but does not display any cleavage activity. Small oligodeoxynucleotides 7 nt in length complementary to various domains of the ribozyme were synthesized (Fig. 15A) and used with 5' end labelled SOFA-ribozyme in the absence (-) or presence (+) of its substrate. The RNA-DNA heteroduplexes were monitored by ribonuclease H (RNase H) hydrolysis, an enzyme that cleaves the RNA strand of such heteroduplexes. A typical gel is shown in Figure 15B.

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The oligodeoxynucleotide complementary to the L3 loop (L3') allowed the detection of a strong band of products in the absence of substrate, indicating that this region was single-stranded, in agreement with a previous report (Ananvoranich & Perreault, *Biochem. Biophys. Res. Comm.* **270**, 600-607, 5 2000). The addition of the substrate also led to the detection of this band at the same intensity, confirming that L3 is still single stranded. This observation is in contradiction to what has been observed in a previous study (Ananvoranich & Perreault, *Biochem. Biophys. Res. Comm.* **270**, 600-607, 2000), but the experiments were performed here under different conditions than in the earlier 10 report. In this work, the oligodeoxynucleotide and the ribozyme were mixed together and incubated for 10 min prior to the addition of RNase H for the same period of incubation. These conditions favour the hybridization of the oligodeoxynucleotide to the L3 loop over the folding of the P1.1 stem that would release the oligodeoxynucleotide. Conversely, the oligodeoxynucleotide 15 complementary to the P4 stem (P4') did not permitted the detection of any products of RNase H hydrolysis, confirming that this region is double-stranded. The oligodeoxynucleotide complementary to the biosensor sequence (BS') permitted the detection of a relatively abundant RNase H product only in the absence of the substrate, indicating that this region was single-stranded within 20 the *off* conformation. Only a trace amount of the hydrolysis product was detected upon the addition of the substrate, showing that in the *on* conformation the biosensor is bound to its substrate and thus is double-stranded. The presence of the oligodeoxynucleotide complementary to the blocker sequence (BL') gave the opposite pattern: no RNase H product was observed in the 25 absence of the substrate, indicating that the blocker sequence was double-

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stranded (with the P1 strand of the ribozyme) within the *off* conformation; while cleavage product was detected in the presence of the substrate, showing that, under these conditions, the blocker was single-stranded. However, a small amount of product was detected, regardless of the length of the oligodeoxynucleotide tested (e.g. slightly longer). We believe this occurs because as this region is central to the species, the RNase H hydrolysis may be limited due to steric hindrance reducing the accessibility to the RNA-DNA heteroduplex. Finally, an oligodeoxynucleotide complementary to the stabilizer (ST') did not allow for the detection of any RNase H products, confirming that this region is double-stranded regardless of the presence or absence of the substrate. In conclusion, the three segments of sequence composing the SOFA module were shown to fold into the expected structure. Moreover, the structure of the blocker and biosensor sequences were observed to be involved in the conformational transition.

15

SOFA-Ribozyme as molecular tools in cultured cells

In order to confirm the great potential of SOFA-ribozymes as molecular tools for gene inactivation systems, a first experiment targeting an HBV derived transcript was performed in cultured cells (Fig. 16). Briefly, the HBV C gene open reading frame was subcloned in the inducible pINDTM vector (Invitrogen) (Fig. 16A). This vector contains five modified ecdysone response elements (E/GREs) and the minimal heat shock promoter for expression of RNA of interest. Using HEK-293 cells that stably express the ecdysone receptor by which the inductor ponasterone A enters the cell, the expression of the targeted

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RNA can be controlled. Either the original or SOFA ribozymes were cloned downstream of the cytomegalovirus (CMV) promoter from a modified pcDNA3 vector (i.e. pm δ Rz; see Fig. 16B). This allowed efficient *in vivo* transcription by RNA polymerase II and ensure localization of the ribozymes in the cytoplasm.

5 Fig. 16C illustrates an autoradiogram of the Northern blot hybridization demonstrating the success of the SOFA- δ Rz activity to diminish the RNA target level. In the presence of the original δ Rz-303, only a weak reduction of the RNA level was observed. However, over 60% of RNA level reduction was observed in the presence of the SOFA⁺- δ Rz-303 version. Conversely, in the presence of a

10 SOFA⁻- δ Rz-303 no reduction was observed. For these three versions of ribozymes, the corresponding inactive version including a mutation of the cytosine in position 47 for an adenosine was synthesized and tested. This mutation allowed the ribozyme to bind its target with the same affinity but it is completely inactive in terms of catalytic activity. Any of these three mutants

15 exhibited cleavage activity *in vivo*. The probing of the β -actin served to normalize the results. More importantly, together, these results confirmed the great potential in cell environment of the SOFA module to activate the cleavage activity solely in the presence of the good substrate.

Flexibility of the SOFA module on δ ribozyme

20 In order to investigate the flexibility of the SOFA module, different versions of the SOFA- δ Rz-303 were synthesized and their cleavage activities were assessed (Figs. 17 and 18). Firstly, the SOFA adapter was moved from the P2 stem to the P4 stem, to obtain a ribozyme called SOFA-down (SOFA-

8Rz-DN, DN for down, SEQ ID NO:84) (Fig. 17A). The SOFA⁺-8Rz-DN cleaved relatively efficiently the transcript (SEQ ID NO:85), although at a reduced level compared to the SOFA⁺-8Rz-303 (Fig 17C). In contrast, the SOFA⁻-8Rz-DN was inactive, as expected (Fig. 17C). Another variation was the construction of a

5 "double" SOFA-ribozyme (SOFA⁺-8Rz-DB, DB for double binding; see Fig. 17B, SEQ ID NO:86). This ribozyme bound the substrate (SEQ ID NO:87) through the formation of three helices involving 32 base pairs. The SOFA⁺ version exhibits a relatively high cleavage activity while the SOFA⁻ did not cleave the substrate. These results illustrate the gains in terms of substrate specificity and

10 "safety lock" action obtained by using the concept of a blocker. It should be noted that the blocker domain of the SOFA module inserted in the L4 loop interacted with the sequence of the J4/2 junction including the C47 of the ribozyme (Figs. 17A and 17B). Moreover, this experiment demonstrated that the position of the SOFA module is not restricted to the P2 stem of the δ ribozyme

15 (upper part); it can also be introduced at the end of the L4 loop (lower part). This conclusion receives additional support from the design of different versions of ribozymes with SOFA modules introduced only into the L4 loop.

In order to demonstrate that the stabilizer stem do not interact with the biosensor or the blocker action, a SOFA-ribozyme lacking this domain (Fig.

20 18A, SEQ ID NO:88) was constructed. The SOFA⁺-8Rz-NS (NS for no stabilizer) cleaved the HBV transcripts (SEQ ID NO:89) to the same extent as the original SOFA⁺-8Rz-303 while the corresponding SOFA⁻-ribozyme (SOFA⁻-8Rz-NS) did not exhibit significant levels of cleavage activity (Fig. 18B). These results showed that the biosensor and blocker domains function independently

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of the presence of the stabilizer domain. However, independent *in vivo* experiments have shown that the stabilizer domain significantly increases the stability of these SOFA-ribozymes.

Summary of the SOFA concept controlling δ ribozymes

5 The concept of a target-dependent module provides for a new generation of biosensorized ribozymes having a significantly improved substrate specificity and efficiency. The *on* conformation implies that a ribozyme with a greater affinity for its substrate subsequently cleaves them faster. Meanwhile, the *off* conformation prevents cleavage of an inappropriate target, acting as a "safety

10 lock". The design of the specific *on/off* adapter was influenced by several factors. First, it is reminiscent of the human immune system, more specifically the cytotoxic T lymphocyte's activation mechanism. The T lymphocytes bind specific cell surface molecules which in turn dictate the T cell's responses. In the same way, the SOFA-ribozyme hybridizes to the RNA target (the activator)

15 and specifically cleaves it. Second, the biosensor also remembers the mechanism of action of an oligodeoxynucleotide acting as facilitator for ribozyme cleavage. However, the linkage of the biosensor directly to the ribozyme permitted a great gain, in terms of cleavage activity, compared to the use of two distinct molecules. Third, the blocker stem was influenced by the

20 TRAP strategy (for Targeted Ribozyme-Attenuated Probe) in which a 3' terminal attenuator anneals to conserved bases in the catalytic core to form the *off* state of a hammerhead ribozyme. The blocker domain of the SOFA module also inactivates the cleavage activity of the ribozyme by binding a sequence that is part of the catalytic core. Finally, the idea of a stabilizer domain that places the

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3'-end of the SOFA module in a double-stranded region originated from the previous demonstration that the P2 stem of the δ ribozyme, which plays the same role in the wild type ribozyme, provides an outstanding stability to this RNA species. In fact, it has been shown that the δ ribozyme was at least an
5 order of magnitude more stable compared to a hammerhead ribozyme in cultured cells. Clearly, the SOFA module is the fruit of a rational design. Using the Systematic Evolution of Ligands by EXponential enrichment (SELEX; Wilson, DS and Szostak, JW *Annu. Rev. Biochem.* 68, 611-647, 1999) approach it would have been impossible to develop this kind of module for a
10 ribozyme.

All the sequence segments that might influence the efficiency of the SOFA module have been decorticated (i.e. the blocker, the biosensor, the stabilizer and the spacer). Initially, the SOFA-ribozyme is in an inactive conformation due to the action of the blocker sequence that formed a stem with
15 the ribozyme's P1 strand, acting as a "safety lock" (Fig. 2). RNase H probing of the ribozyme alone supports the hypothesis that the blocker is engaged in a double-stranded region, while the biosensor sequence remains single stranded and accessible (Fig. 15). The optimal size for the blocker sequence was determined to be 4 nucleotides (see Fig. 12). Smaller blockers did not
20 sufficiently prevent the ribozyme's activity, while longer blockers appeared to lock the ribozyme in its inactive conformation (in addition to leading, in some cases, to self-cleavage resulting from formation of a structure reminiscent of that of a *cis*-acting ribozyme). Moreover, we observed that the action of the blocker sequence of various SOFA-ribozymes couldn't be correlated with the

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identity of the residues composing this segment. Thus, a higher GC content in the blocker was not responsible for the lower activity of some of the SOFA-ribozymes. Regardless, there was competition between the blocker (4 bp) and the substrate (7 bp) for the P1 sequence; therefore, a higher GC content on one
5 strand would be counterbalanced by a higher concentration on the other strand.

Since the idea of a blocker stem was inspired by the targeted ribozyme-attenuated probe (TRAP) designed for the hammerhead ribozyme, the comparison of the latter with SOFA appears to be important. Both approaches are based on the inhibition of ribozyme action due to the presence of a *cis*-
10 acting antisense sequence. With TRAP, the presence of an oligodeoxynucleotide complementary to both this *cis*-acting sequence and a portion of the ribozyme activates the ribozyme. Consequently, there is no interaction between the oligodeoxynucleotide and the substrate. Conversely, with the SOFA module the action of the blocker is removed following the binding
15 of the biosensor to the substrate. As a result there is no requirement for a third partner. The TRAP ribozyme has demonstrated an activation of cleavage of as much as 1760 fold, with an average of more than 250 fold. In the case of the SOFA-ribozyme higher than a 15 000 fold increase has been observed, with an average of more than 800 fold. In other words, the SOFA system brings a two
20 order of magnitude increase in the specificity to the ribozyme's action. Thus, the SOFA concept appears to be a more efficient mode of increasing the substrate specificity of a ribozyme.

In the presence of the desired substrate, the biosensor binds the complementary substrate sequence, leading in the release of the ribozyme's P1
25 stem from the blocker (Fig. 2). The RNase H probing of the SOFA-ribozyme-

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substrate complex strongly suggest that the biosensor is base-paired with the substrate; while the blocker becomes susceptible to RNase H hydrolysis, indicating that it is single-stranded (Fig. 15). Kinetic experiments have previously shown the optimal size of the biosensor to be 10 nucleotides. We demonstrated that each SOFA-ribozyme in our collection efficiently cleaved only the substrate containing the sequence complementary to its biosensor (see Fig. 10). Substrates that included sequence with several mutations in the binding region of the biosensor were poorly cleaved. Under single turnover conditions ($[Rz] \gg [S]$), which should favour cleavage of even imperfectly base-paired substrates, only a residual rate of cleavage was observed. A similar conclusion was obtained when investigating a biosensor possessing a small number of mutations (Fig. 11). As expected, the decrease in the cleavage activity was inversely proportional to the number of mutations (ranging from 4 to 106 fold smaller in terms of k_{obs}). In the presence of a single mutation the reduction was estimated to be from 4 to 15 fold. However, the determination of the kinetic parameters for some mutated ribozymes led us to observe larger effects in terms of the second order rate constant (k_{cat}/K_M 25 fold smaller). It should be noted that most of these experiments were performed using small substrates. A more important effect was observed with several of these SOFA-ribozymes when they were tested for the cleavage of the longer HBV-derived transcript (1190 nt). More importantly, a reduction of approximately one order of magnitude is probably sufficient for the ribozyme to be able to discriminate between two substrates, while a smaller difference would require additional precautions in order to ensure the substrate specificity for SOFA-ribozyme

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based cleavage in a cell. These data demonstrate the potential of the biosensor to significantly improve the substrate specificity of a ribozyme.

In both the inactive and active conformations, the SOFA-ribozymes harbour a stabilizer stem that joins the sequence found at the 5' and 3' ends into a stem (Fig. 2). This structure was confirmed by RNase H probing (Fig.15). In terms of mechanism, it appears clear that the stabilizer does not have an active role in the SOFA module (see Fig. 14) other than the improvement of the structure's stability.

Finally, the length of the spacer sequence was investigated. The spacer sequence is not part of the SOFA-module, but it is an important parameter that influences the cleavage level. The spacer is the sequence located between the substrate P1 strand domain and the sequence complementary to the biosensor (Fig. 2). It was shown that a minimal spacer of at least one nucleotide was preferable. Moreover, short spacer sequences (1 to 5 nucleotides) appeared to have higher levels of cleavage than did longer ones (see Fig. 13). Most likely the binding of the biosensor favours the subsequent formation of the P1 stem between the ribozyme and the substrate when the spacer is short.

Together, these experiments with SOFA- δ Rz-303 yield a better understanding of the contribution of each of the different domains of the SOFA module. Data obtained with other ribozymes supports the hypothesis that our findings are not restricted to SOFA- δ Rz-303, but rather can be applied to other SOFA- δ Rzs.

This new approach provides a highly specific and improved tool with a lot of potential in both functional genomics and gene therapy. In terms of

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specificity, considering only the base pairs formed during the two binding steps between a SOFA- δ ribozyme and its substrate (7 bp for P1 binding stem + 10 bp for the biosensor stem), a single site should exist per 1.7×10^{10} bases (4^{17}). The human genome is composed of 3×10^9 base pairs, of which ~5% form mRNAs (i.e. 1.5×10^8 bases). Therefore, the substrate specificity of a SOFA- δ ribozyme is greater than 100 fold superior to what is needed to hit one site. This initiative provides confidence in the use of ribozymes in gene therapy and functional genomic applications, even if a mismatch is tolerated in the biosensor.

10 SOFA module controlling other nucleic acid species

This is the first report of a ribozyme of an endonuclease-type that harbors a target-dependent module that is activated by a nucleic acid RNA substrate and then cleaves this molecule. This new concept offers great promise and should prompt a new "taking off" of the ribozyme field. Furthermore, this concept can be substantially extended to other RNA drug-based molecules that aim to cleave RNA molecules. For example, Fig. 19 illustrates one way to adapt the SOFA module to a cleaving hammerhead ribozyme, a cleaving hairpin ribozyme, a ligating hairpin ribozyme, or a cleaving DNzyme (i.e. a DNA molecule that possesses catalytic ability) (SE ID NO:90-93 and 98-101). Both the off and on (upon addition of the substrate, SEQ ID NO:94-97) conformations are illustrated. The biosensor (BS) and blocker (BL) domains are in grey. The substrates are squared. Since all motifs possess single-stranded extremities, we proposed a similar design in which the blocker is at one end while the biosensor is at the other end. This means that the SOFA module is split in two

pieces (the blocker and the biosensor), each one with a specific function. The same concept of *off* and *on* conformation depending on the presence of the desired target is respected.

Moreover, a proof-of concept has been performed with the DNzyme.

5 Cleavage assay were performed using a 5'-end ^{32}P -labelled substrate (S) of 46 nucleotides that generates a 5'-product of 23 nucleotides. The DNzyme were purchased as DNA oligonucleotide and used directly in the experiments. The reactions were performed and illustrated in Fig. 20, which shows an autoradiogram of a 6% denaturing PAGE gel of the cleavage assays. The

10 substrate was incubated alone (lane 1), with a DNzyme (lane 2), or with different versions of SOFA-DNzyme. The SOFA module were assessed using separately either a good or irrelevant biosensor of 14 nucleotides in size (lanes 3 and 4, respectively), and a blocker sequence of 10 nucleotides (lane 5). Finally, SOFA module (i.e. including biosensor and blocker) were tested using

15 both an appropriate biosensor (i.e. complementary to the substrate; SOFA⁺-DNzyme) and an irrelevant biosensor (i.e. not complementary to the substrate; SOFA⁻-DNzyme) (lanes 6 and 7, respectively). The original DNzyme cleaved a small radiolabelled substrate while a version harboring the blocker sequence was inactive (lanes 2 and 3). A SOFA⁺-DNzyme (i.e. with a blocker and a

20 biosensor with the appropriate sequence to target the substrate) exhibited cleavage activity while not with a SOFA⁻ (i.e. biosensor not complementary to the substrate). This shows that the SOFA concept is not restricted to δRz , and more generally to RNA.

Similarly, Fig. 21 shows an application to the silencing RNA (siRNA) which is another RNA based approach for gene-inactivation (SEQ ID NO:102 to 104). The same concept of *off* and *on* conformation depending on the presence of the desired target is respected.

5 Thus, this technology can also be applied to other fields such as to siRNA or any other RNA implicated in a specific disease, its development or its spreading. By adapting the biosensor sequence and the blocker sequence, the SOFA can be made specific for such siRNA or other nucleic acid, acting as an *on/off* switch and improving substrates specificity, even if no enzymatic activity
10 is involved such as with ribozymes. The present invention can thus increase the popularity of siRNA which are these days often investigated as being a possible treatment for some conditions, but in life so far are not so often used due to their lack of specificity or to their immunogenicity. The present invention can also be used with success in treatment for breast cancer to prevent
15 transcription of the faulty genes, or in treatment of Alzheimer, preventing accumulation of irrelevant RNA.

WHAT IS CLAIMED IS:

1. A target-dependent nucleic acid adapter adapted to be matched to a substrate comprising a target sequence, said adapter having a nucleic acid sequence comprising linked together:

- i) a blocker stem sequence complementary to a portion of said nucleic acid sequence; and
- ii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the nucleic acid sequence for said target sequence,

wherein in absence of the target sequence of said substrate, said blocker stem sequence forms an intramolecular stem with said nucleic acid sequence linked thereto, preventing exposition of the nucleic acid sequence, thus locking said nucleic acid sequence of the adapter in an inactive conformation, and, in presence of said target sequence of said substrate, said biosensor sequence forming conventional Watson-Crick base pairs with said target sequence and said blocker stem sequence dissociating from the intramolecular stem, thus exposing said nucleic acid sequence of said adapter in an active conformation.

2. The target-dependent nucleic acid adapter of claim 1, further comprising a nucleic acid sequence forming a stabilizing stem, whereby the 3' end of the nucleic acid sequence of the adapter is paired up with said nucleic acid sequence forming an intramolecular stem at the 3' end of said nucleic acid

sequence, thus preventing or reducing degradation of said nucleic acid sequence.

3. The target-dependent nucleic acid adapter of claim 2, wherein the stabilizing stem has two complementary strands, a first strand of which is linked to the 5'-end of the biosensor sequence, and a second strand of which is complementary to the first strand and is adapted to be linked at its 5'-end to the 3'-end of the nucleic acid sequence, thus preventing exposure of a single stranded 3'-end sequence susceptible to degradation by cellular nuclease.
4. The target-dependent nucleic acid adapter of claim 3, wherein the first strand of the stabilizing stem has a sequence as set forth from residue 4 to 11 of SEQ ID NO:1 and the second strand of the stabilizing stem has a sequence as set forth from residue 96 to 103 of SEQ ID NO:1.
5. The target-dependent nucleic acid adapter of claim 1, wherein the blocker stem sequence has a sequence specific for a ribozyme.
6. The target-dependent nucleic acid of claim 5, wherein the ribozyme is ribozyme delta.
7. The target-dependent nucleic acid adapter of claim 1, wherein the biosensor has a sequence as set forth from residue 15 to 29 of SEQ ID NO:1.
8. The target-dependent nucleic acid adapter of claim 1, wherein the blocker stem sequence has a sequence as set forth from residue 30 to 33 of SEQ ID NO:1.

9. The target-dependent nucleic acid adapter of claim 1, having a sequence as illustrated in Figure 2A.

10. The target-dependent nucleic acid adapter of claim 1, wherein the blocker stem sequence is linked to the biosensor.

11. A method for improving specificity of a nucleic acid sequence for a target sequence, said method comprising the steps of attaching to said nucleic acid sequence a target-dependent nucleic acid adapter having a nucleic acid sequence comprising:

- i) a blocker stem sequence complementary to a portion of said nucleic acid sequence; and
- ii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the nucleic acid sequence for said target sequence,

wherein in absence of the target sequence of said substrate, said blocker stem sequence forms an intramolecular stem with said nucleic acid sequence linked thereto, preventing exposition of the nucleic acid sequence, thus locking said nucleic acid sequence of the adapter in an inactive conformation, and, in presence of said target sequence of said substrate, said biosensor sequence forming conventional Watson-Crick base pairs with said target sequence and said blocker stem sequence dissociating from the intramolecular stem, thus exposing said nucleic acid sequence of said adapter in an active conformation.

12. The method of claim 11, further comprising a nucleic acid sequence forming a stabilizing stem, whereby the 3' end of the nucleic acid sequence of the adapter is paired up with said nucleic acid sequence forming an intramolecular stem at the 3' end of said nucleic acid sequence, thus preventing or reducing degradation of said nucleic acid sequence.
13. The method of claim 12, wherein the stabilizing stem has two complementary strands, a first strand of which is linked to the 5'-end of the biosensor sequence, and a second strand of which is complementary to the first strand and is adapted to be linked at its 5'-end to the 3'-end of the nucleic acid sequence, thus preventing exposure of a single stranded 3'-end sequence susceptible to degradation by cellular nuclease.
14. The method of claim 13, wherein the first strand of the stabilizing stem has a sequence as set forth from residue 4 to 11 of SEQ ID NO:1 and the second strand of the stabilizing stem has a sequence as set forth from residue 96 to 103 of SEQ ID NO:1.
15. The method of claim 11, wherein the blocker has a sequence specific for a ribozyme.
16. The method of claim 15, wherein the ribozyme is ribozyme delta.
17. The method of claim 16, wherein the biosensor has a sequence as set forth from residue 15 to 29 of SEQ ID NO:1.
18. The method of claim 11, wherein the blocker stem sequence has a sequence as set forth from residue 30 to 33 of SEQ ID NO:1.

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19. The method of claim 11, wherein the adapter has a sequence as illustrated in Figure 2A.

20. The method of claim 11, wherein the blocker stem sequence is linked to the biosensor.

21. A method for turning *on* or *off* an enzymatic activity of a nucleic acid molecule having an enzymatic activity, said method comprising the steps of attaching to said nucleic acid molecule a nucleic acid target dependent adapter having a nucleic acid sequence comprising,:

- i) a blocker stem sequence complementary to a portion of said nucleic acid sequence; and
- ii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the nucleic acid sequence for said target sequence,

wherein in absence of the target sequence of said substrate, said blocker stem sequence forms an intramolecular stem with said nucleic acid sequence linked thereto, preventing exposition of the nucleic acid sequence, thus locking said nucleic acid sequence of the adapter in an inactive conformation, turning off the enzymatic activity and, in presence of said target sequence of said substrate, said biosensor sequence forming conventional Watson-Crick base pairs with said target sequence and said blocker stem sequence dissociating from the intramolecular stem, thus exposing said nucleic acid sequence of said adapter in an active conformation, turning on the enzymatic activity.

22. The method of claim 21, further comprising a nucleic acid sequence forming a stabilizing stem, whereby the 3' end of the nucleic acid sequence of the adapter is paired up with said nucleic acid sequence forming an intramolecular stem at the 3' end of said nucleic acid sequence, thus preventing or reducing degradation of said nucleic acid sequence.
23. The method of claim 22, wherein the stabilizing stem has two complementary strands, a first strand of which is linked to the 5'-end of the biosensor, and a second strand of which is complementary to the first strand and is adapted to be linked at its 5'-end to the 3'-end of the nucleic acid molecule, thus preventing exposure of a single stranded 3'-end sequence susceptible to degradation by cellular nuclease.
24. The method of claim 23, wherein the first strand of the stabilizing stem has a sequence as set forth from residue 4 to 11 of SEQ ID NO:1 and the second strand of the stabilizing stem has a sequence as set forth from residue 96 to 103 of SEQ ID NO:1.
25. The method of claim 21, wherein the blocker has a sequence specific for a ribozyme.
26. The method of claim 25, wherein the ribozyme is ribozyme delta.
27. The method of claim 26, wherein the biosensor has a sequence as set forth from residue 15 to 29 of SEQ ID NO:1.
28. The method of claim 21, wherein the blocker stem sequence has a sequence as set forth from residue 30 to 33 of SEQ ID NO:1.

29. The method of claim 21, wherein the adapter has a sequence as illustrated in Figure 2A.

30. The method of claim 21, wherein the blocker stem sequence is linked to the biosensor.

31. The method of claim 26, wherein the nucleic acid molecule attached to said adapter is ribozyme delta.

32. A target-specific activatable/deactivatable ribonuclease adapted to be matched to a substrate comprising a target sequence, said ribonuclease having a nucleic acid sequence comprising linked together:

- i) a ribonuclease sequence, or an active fragment thereof;
- ii) a blocker stem sequence complementary to a portion of said ribonuclease sequence, said blocker sequence being linked upstream of the ribonuclease sequence; and
- iii) a biosensor sequence having a sequence complementary to said target sequence, said biosensor improving the specificity of the ribonucleic acid sequence for said target sequence, said biosensor being linked to the blocker sequence,

wherein in absence of the target sequence of said substrate, said blocker sequence forms an intramolecular stem with the ribonuclease sequence linked thereto, thus locking said ribonuclease in an inactive conformation, and, in presence of the target sequence of said substrate, said biosensor sequence forming conventional Watson-Crick base pairs with said target sequence and said blocker stem sequence dissociating from the

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intramolecular stem, thus exposing said ribonuclease in an active conformation.

33. The target-specific activatable/deactivatable ribonuclease of claim 32, further comprising sequences forming a stabilizing stem, whereby the 3' end of the adapter so linked to said ribonucleic acid sequence is paired up, thus preventing or reducing degradation of said ribonucleic acid sequence.

34. The target-specific activatable/deactivatable ribonuclease of claim 33, wherein the stabilizing stem has two complementary strands, a first strand of which is linked to the 5'-end of the biosensor, and a second strand of which that is complementary to the first strand and that is adapted to be linked at its 5'-end to the 3'-end of the ribonuclease sequence, thus preventing exposure of a single stranded 3'-end sequence susceptible to degradation by cellular nuclease.

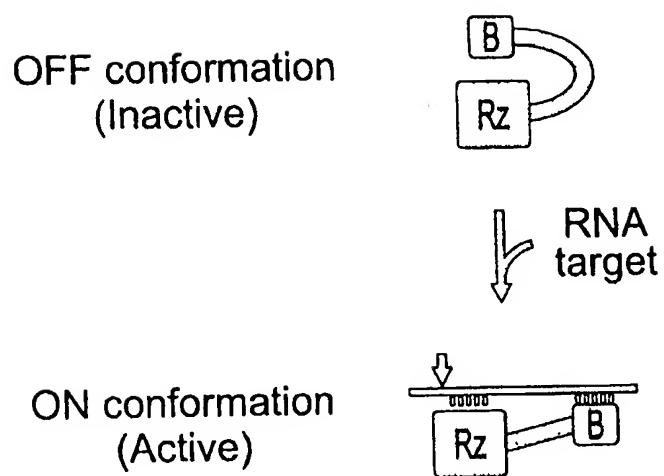
35. The target-specific activatable/deactivatable ribonuclease of claim 34, wherein the first strand of the stabilizing stem has a sequence as set forth from residue 4 to 11 of SEQ ID NO:1 and the second strand of the stabilizing stem has a sequence as set forth from residue 96 to 103 of SEQ ID NO:1.

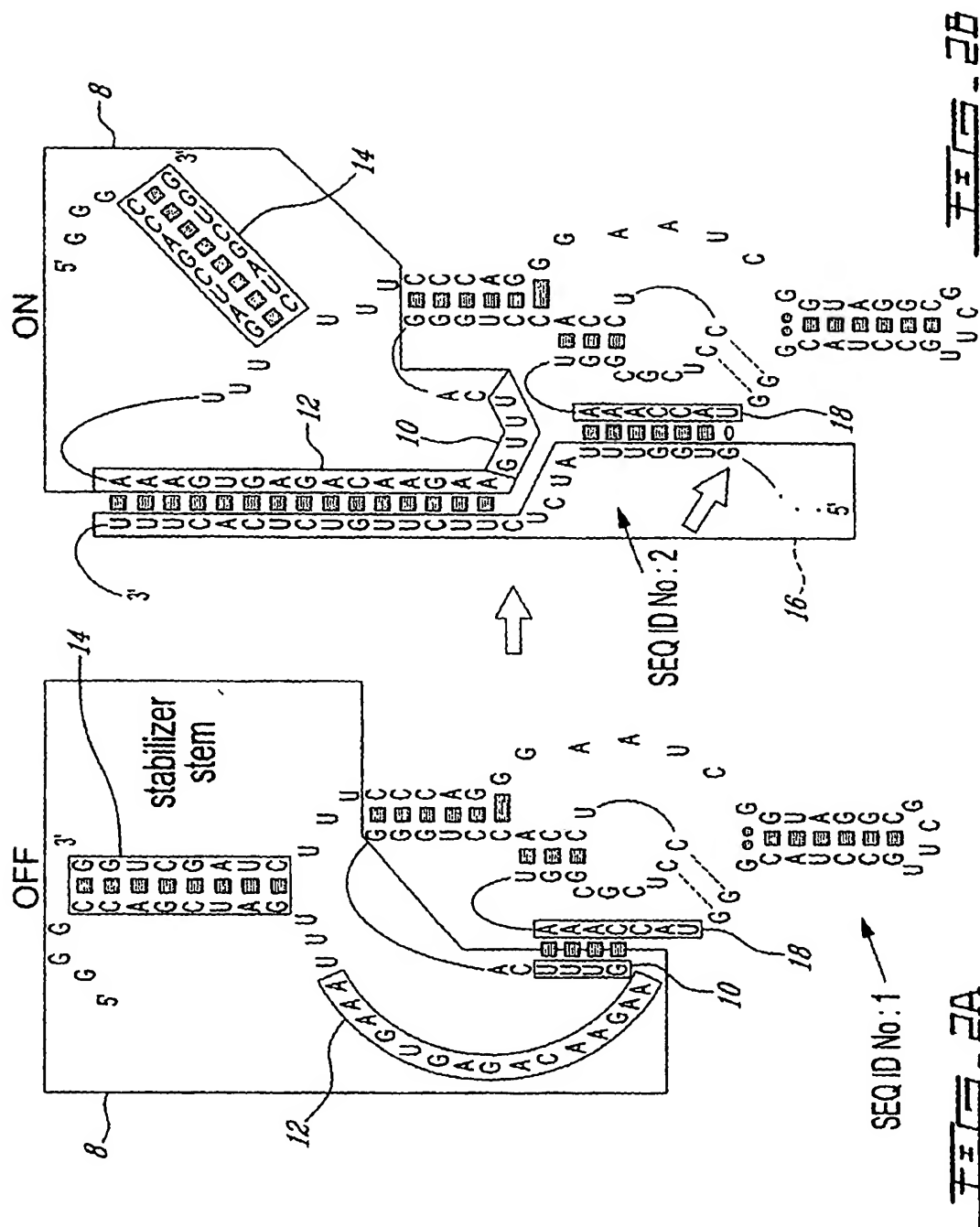
36. The target-specific activatable/deactivatable ribonuclease of claim 32, wherein the blocker stem sequence has a sequence specific for a ribozyme.

37. The target-specific activatable/deactivatable ribonuclease of claim 36, wherein the ribozyme is ribozyme delta.

38. The target-specific activatable/deactivatable ribonuclease of claim 32, wherein the biosensor has a sequence as set forth from residue 15 to 29 of SEQ ID NO:1.
39. The target-specific activatable/deactivatable ribonuclease of claim 32, wherein the blocker stem sequence has a sequence as set forth from residue 30 to 33 of SEQ ID NO:1.
40. The target-specific activatable/deactivatable ribonuclease of claim 32, having a sequence as illustrated in Figure 2A.

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FIG. 1



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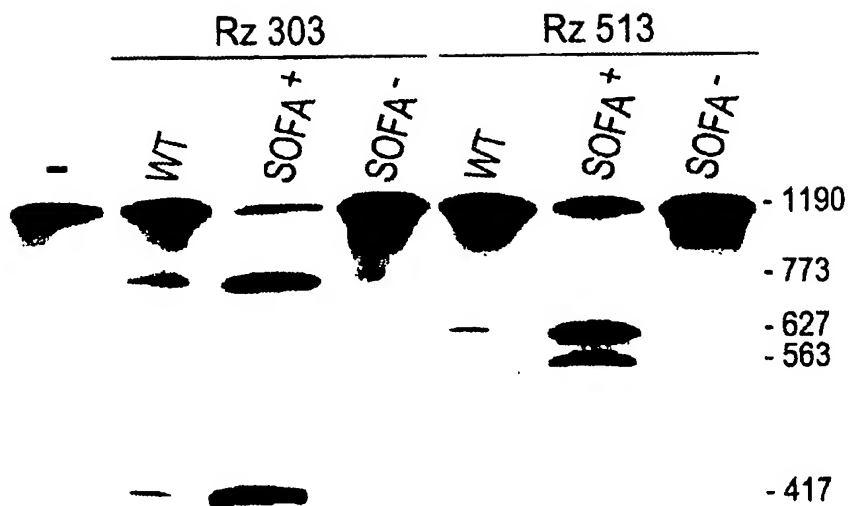


Fig. 3

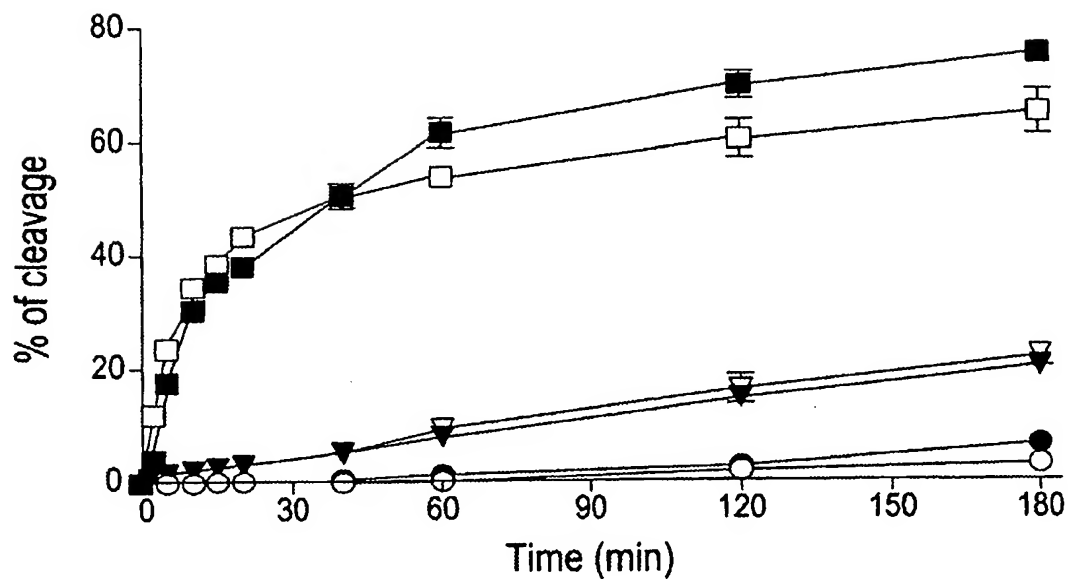
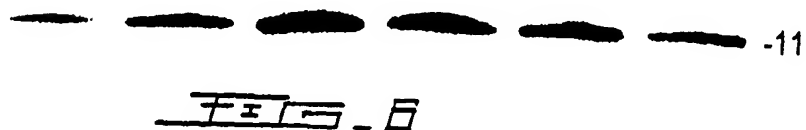
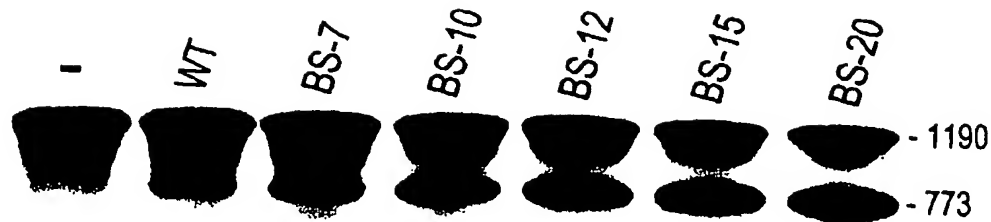
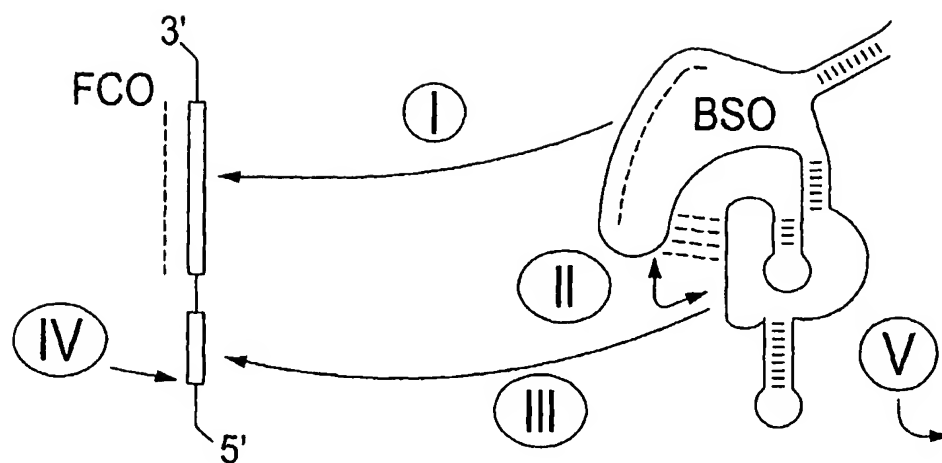
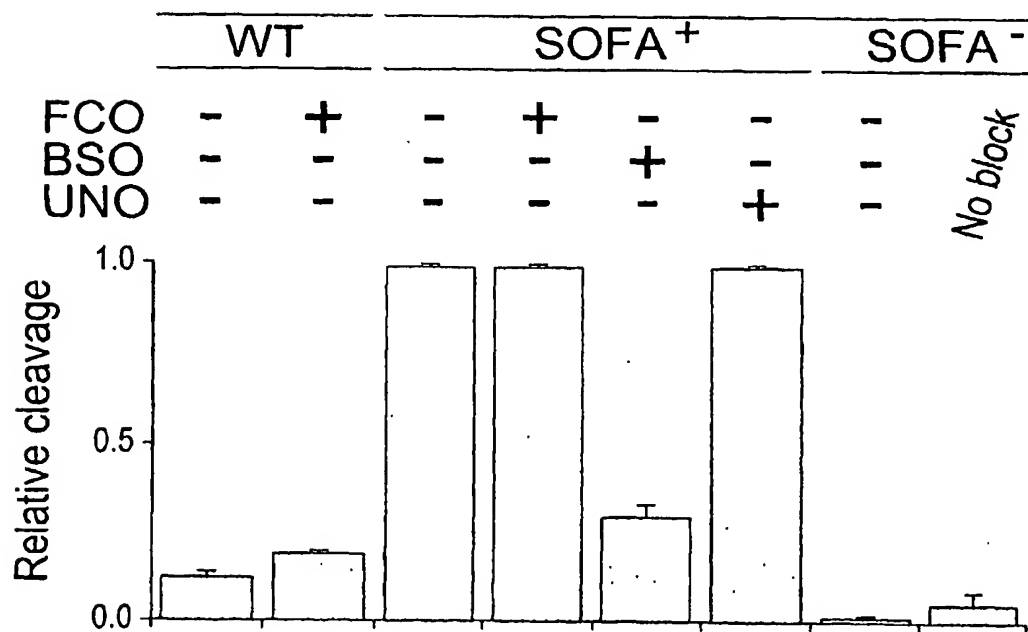


Fig. 4

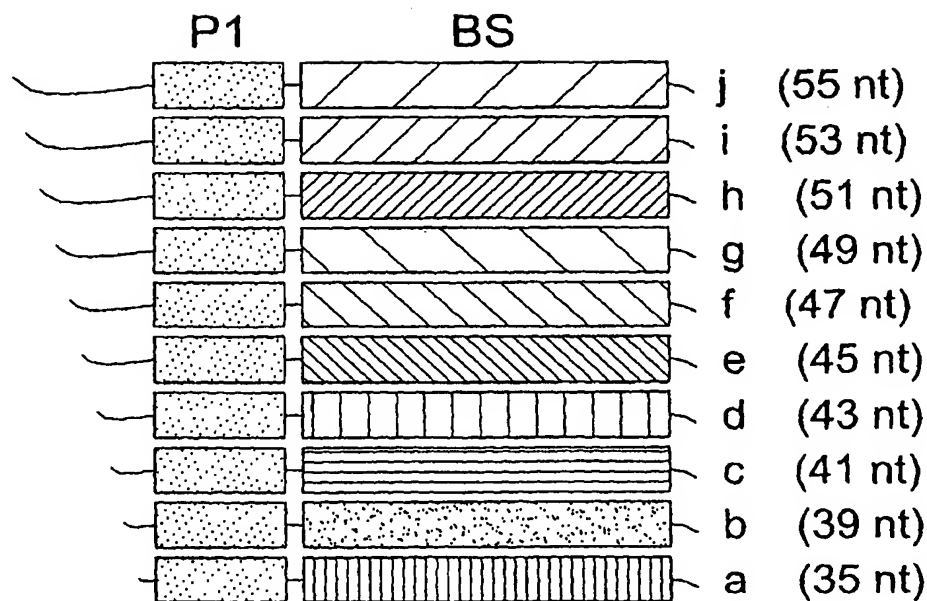
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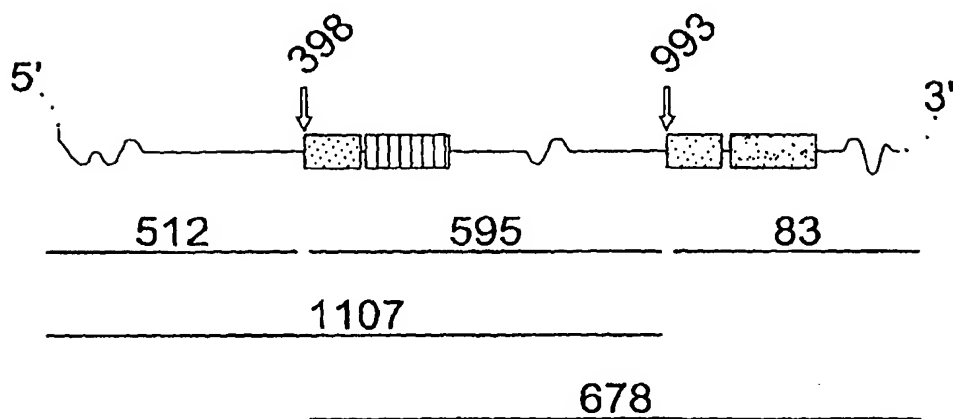
FIG. 7AFIG. 7B

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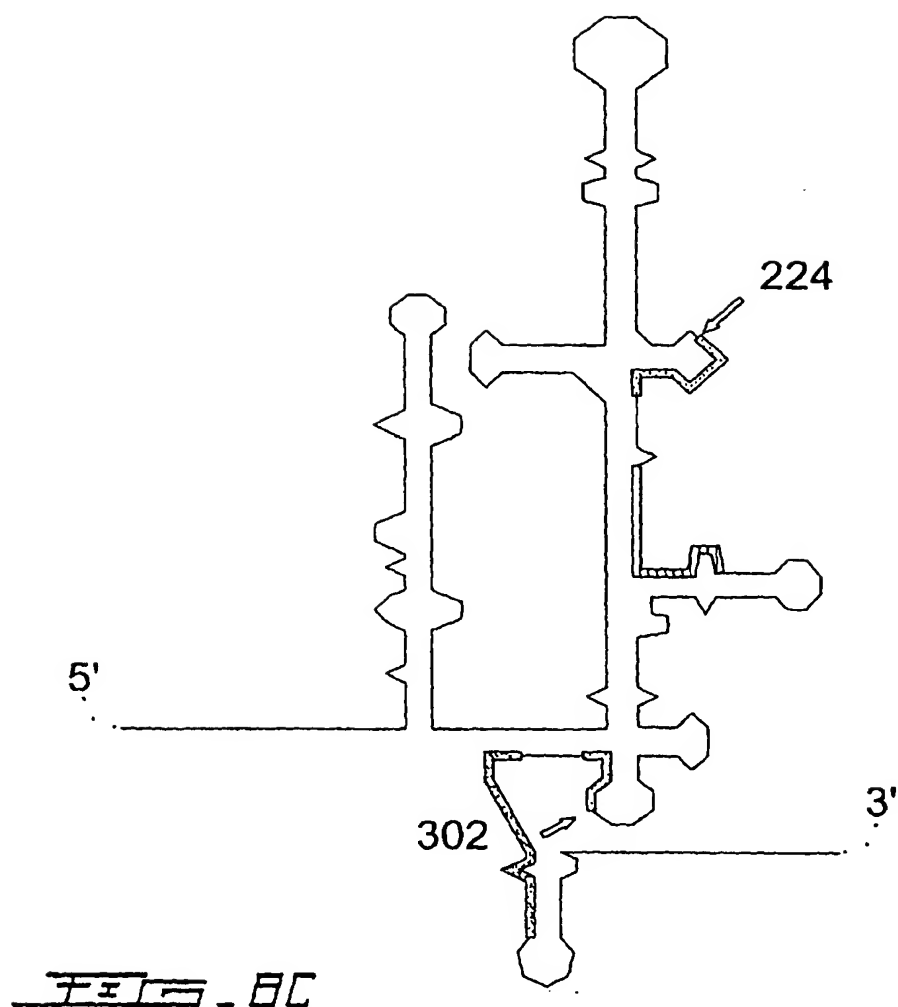
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HBV RNA

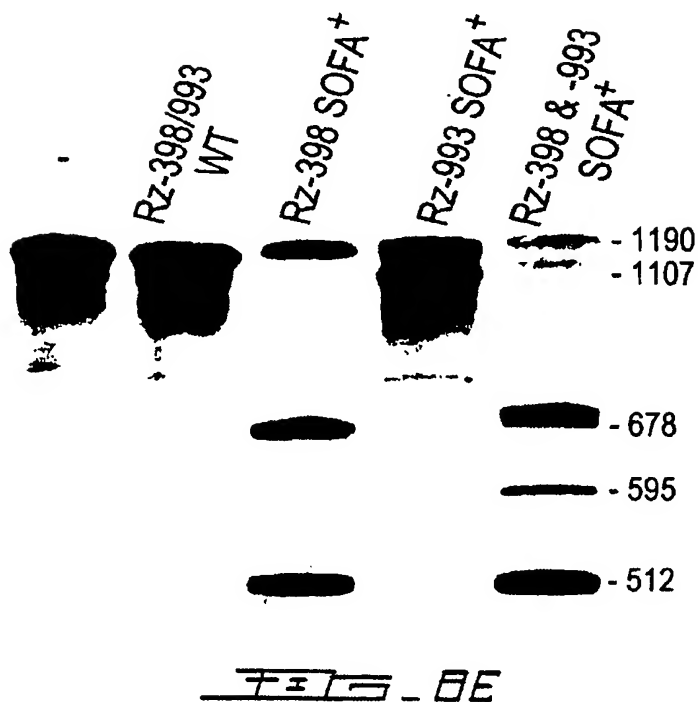
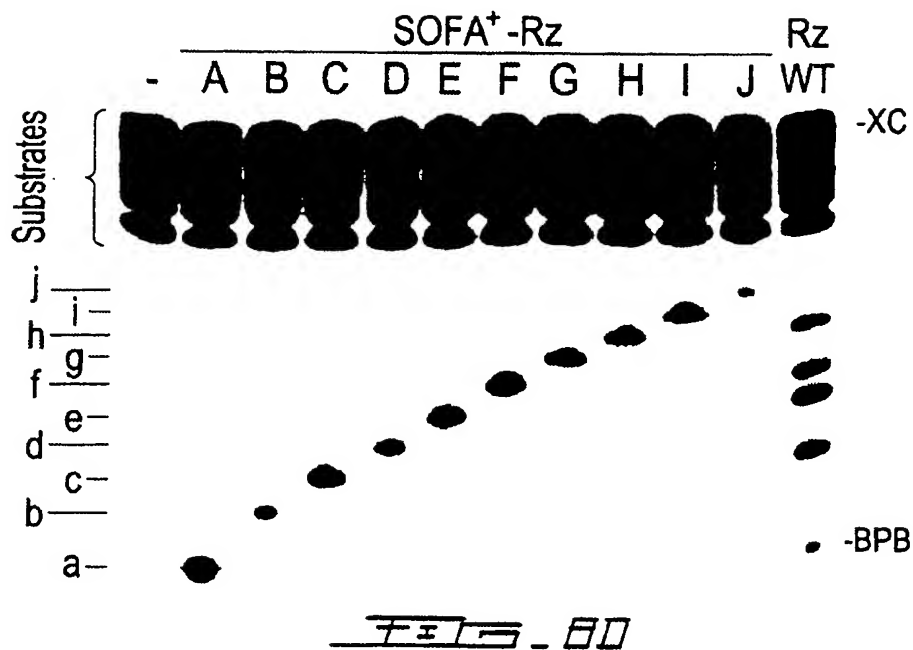


FEI - BA

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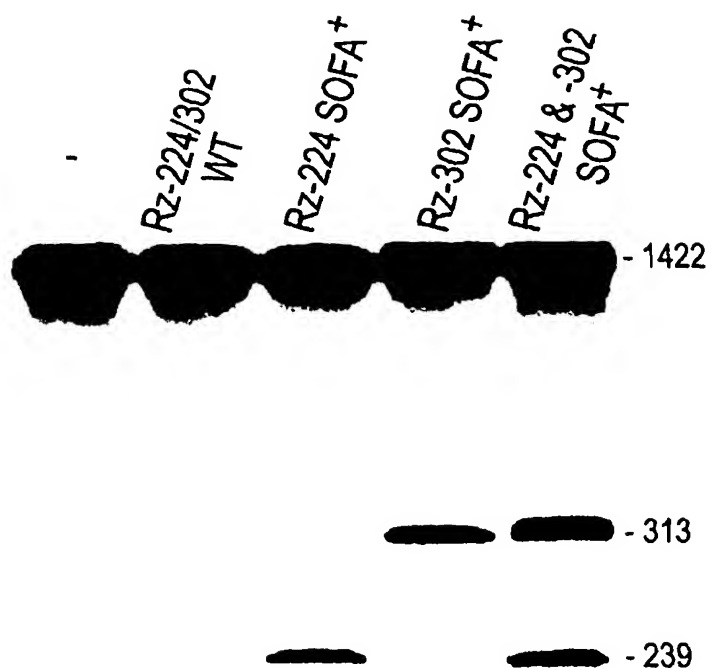


FIG. 10 - BF

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5' ~~~~~	↓	HBV 303	GGCAACTCTTGTTGTTT	<u>P1</u>	BS	CACATTTCTTGCTCTCACTTTTGGAA ~~~~~ 3'	SEQ ID No : 36
5' ~~~~~	↓	HBV 513	CTCAATCGCGCGT	<u>P1</u>	BS	CGCAGAAAGATCTCAATCTCGGGAATCTC ~~~~~ 3'	SEQ ID No : 37
5' ~~~~~	↓	HBV 398	CCAGCTTATAGACCA	<u>P1</u>	BS	CCAAATGCCCCCTATCCCTATCAACACTT ~~~~~ 3'	SEQ ID No : 38
5' ~~~~~	↓	HBV 993	ATTCTTTCCGACCA	<u>P1</u>	BS	CCAGTTGGATCCAGCCCTTCAGAGCAAA ~~~~~ 3'	SEQ ID No : 39
5' ~~~~~	↓	HCV 224	AGATTGGGCGTG	<u>P1</u>	BS	CCCCCGCGAGACTGCTAGCCGAGTAGTGT ~~~~~ 3'	SEQ ID No : 40
5' ~~~~~	↓	HCV 302	GTGCTTGGAGTG	<u>P1</u>	BS	CCCCCGGAGGTCTCGTAGACCGTGCACCA ~~~~~ 3'	SEQ ID No : 41

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SEQ ID	No 42 No 50	No 43 No 51	No 44 No 52	No 45 No 53
	aA C - G U - A C - G U - A G - C U - A U - A C - G U - A U - A	bB U - A C - G U - A G - C A - U C - G U - A C - G U - A A - U	cC C - G A - U A - U G - C U - A C - G G - C U - A G - C A - U	dD U - A C - G C - G U - A A - U U - A G - C C - G C - G C - G
SEQ ID	No 46 No 54	No 47 No 55	No 48 No 56	No 49 No 57
	eE U - A C - G A - U G - C A - U C - G C - G U - A A - U G - C	fF C - G C - G G - C U - A A - U U - A U - A A - U G - C U - A	gG U - A U - A C - G A - U A - U C - G C - G G - C C - G U - A	hH C - G A - U U - A G - C A - U G - C U - A A - U C - G G - C

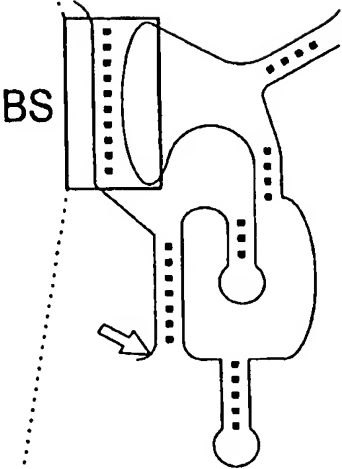
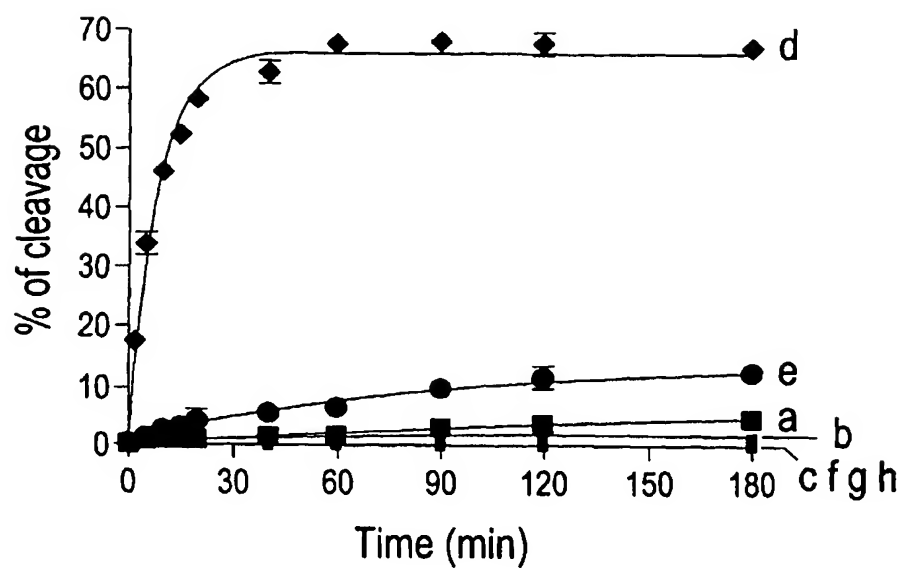
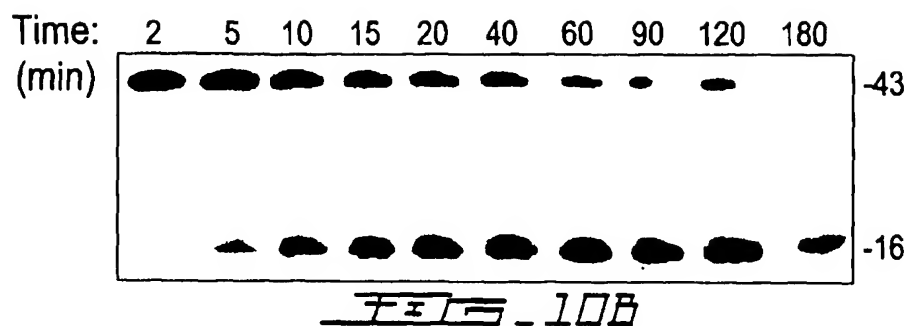


FIG. 10A

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FIS-10C

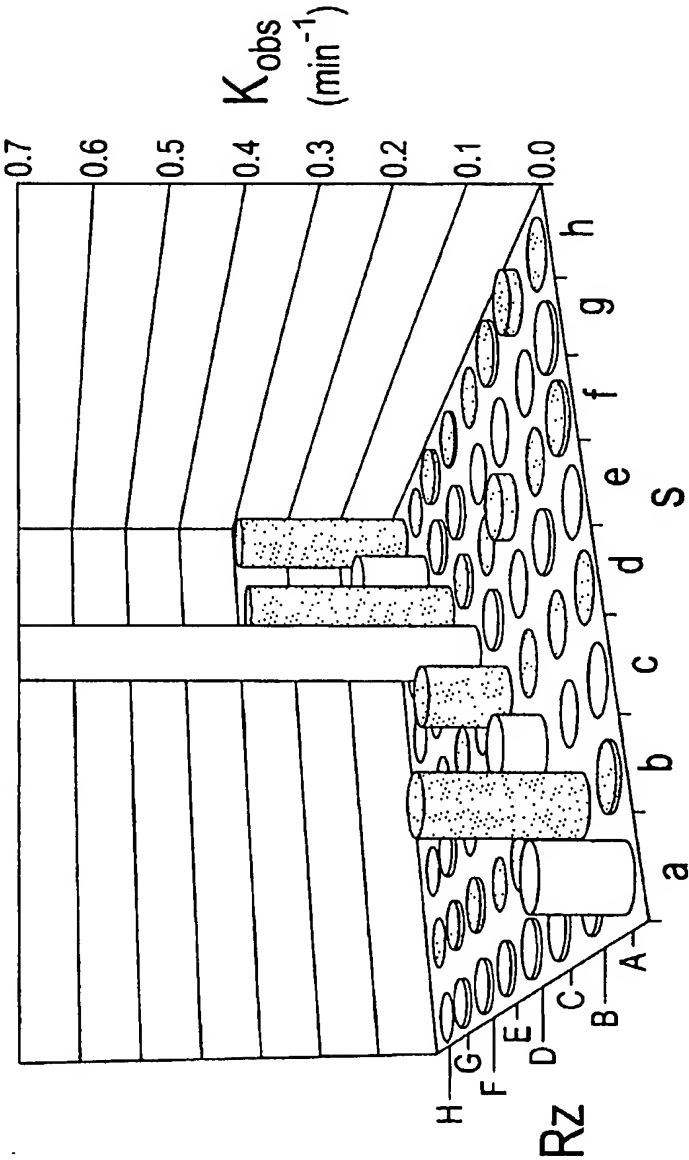


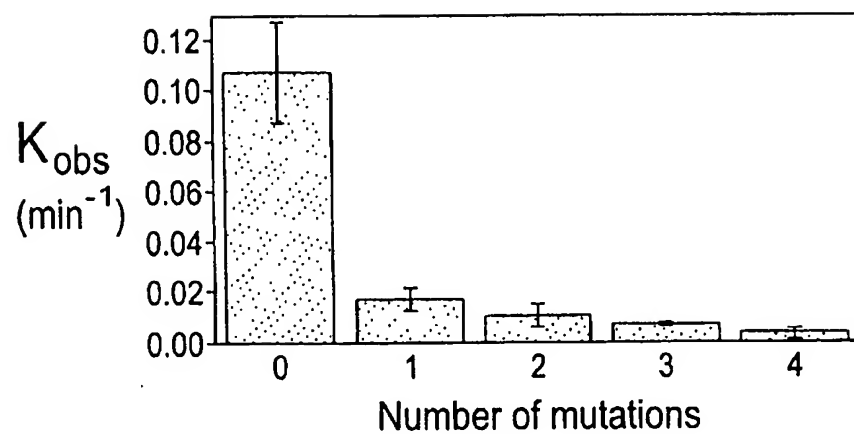
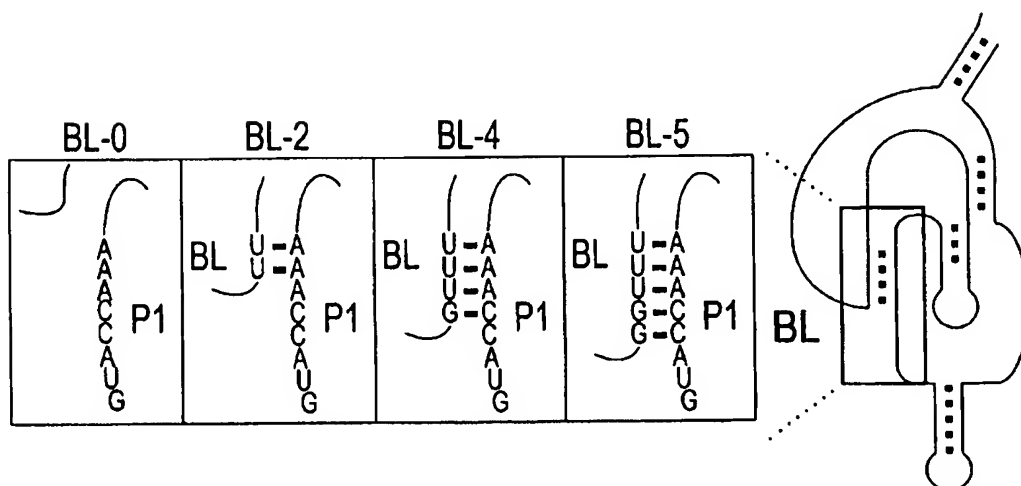
FIG. 100

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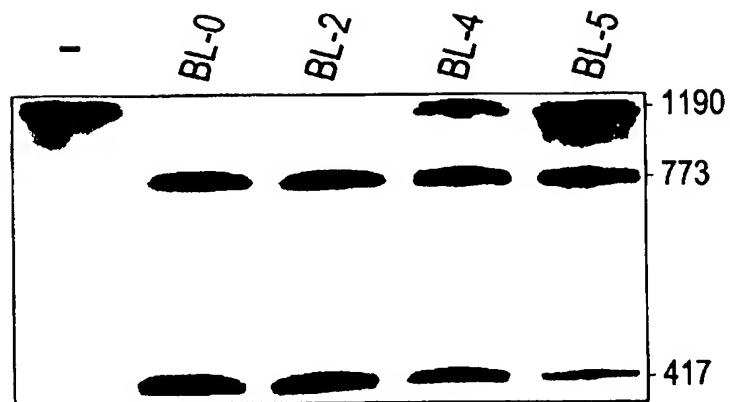
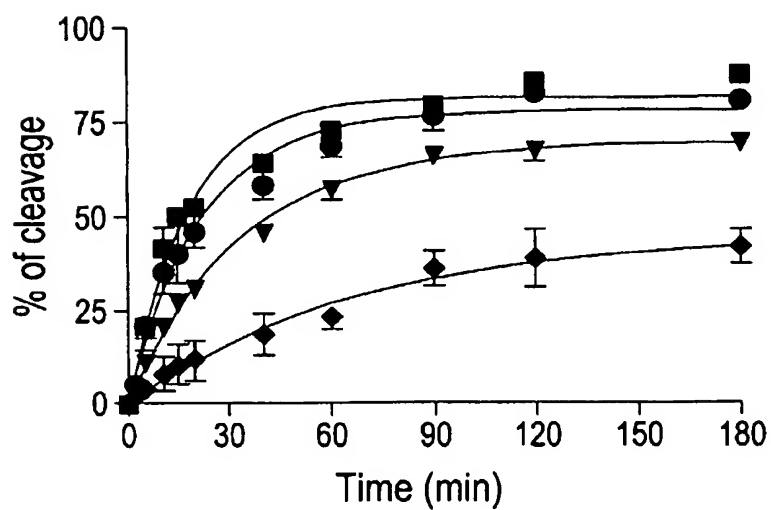
Ribozyme BS		K_{obs} (min^{-1})	
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	□AGACAAGAA	0.029	SEQ ID No : 59
	G□GACAAGAA	0.022	SEQ ID No : 60
	G□GACAAGAA	0.022	SEQ ID No : 61
	GA□ACAAGAA	0.020	SEQ ID No : 62
	GAG□CAAGAA	0.012	SEQ ID No : 63
1	GAGAGAAAGAA	0.022	SEQ ID No : 64
	GAGAC□AGAA	0.011*	SEQ ID No : 65
	GAGAC□AGAA	0.013	SEQ ID No : 66
	GAGACA□GAA	0.007	SEQ ID No : 67
	GAGACAAG□AA	0.016	SEQ ID No : 68
	GAGACAAG□A	0.014*	SEQ ID No : 69
	GAGACAAG□A	0.014	SEQ ID No : 70
	GAGACAAGAA□	0.020	SEQ ID No : 71
	G□GACAAG□A	0.013	SEQ ID No : 72
2	GAGAG□AGAA	0.015*	SEQ ID No : 73
	GAGAC□AG□A	0.007*	SEQ ID No : 74
	GAGAC□AG□A	0.006	SEQ ID No : 75
	□□GAC□AGAA	0.006	SEQ ID No : 76
3	G□GAC□AG□A	0.007	SEQ ID No : 77
	GAGAC□AC□A	0.007	SEQ ID No : 78
	□□□ACAAGAA□	0.006	SEQ ID No : 79
4	G□GAC□AG□□	0.001	SEQ ID No : 80
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FIG. 11A

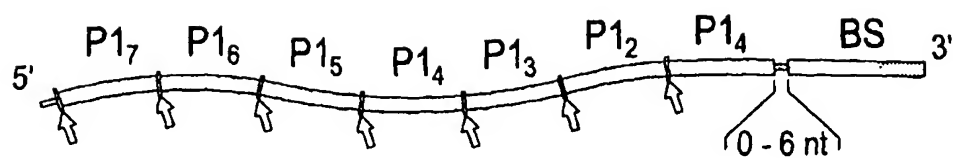
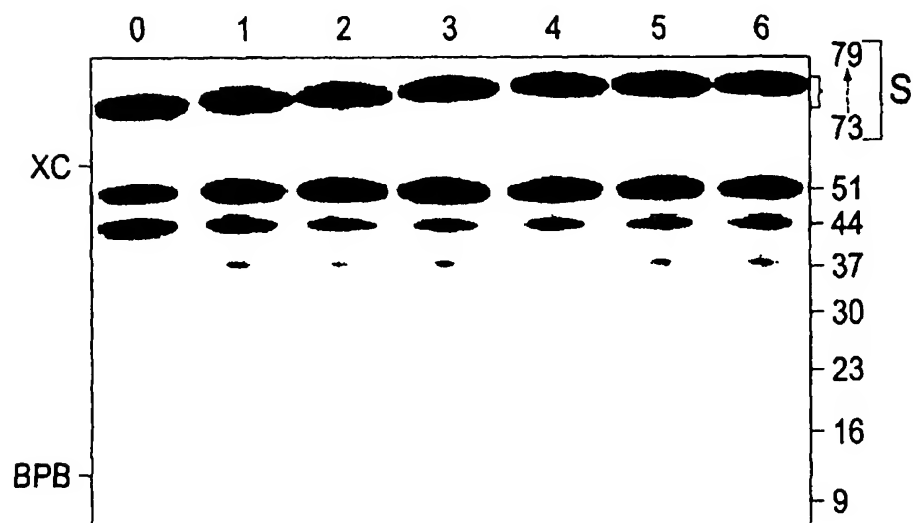
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FIG. 11BFIG. 12A

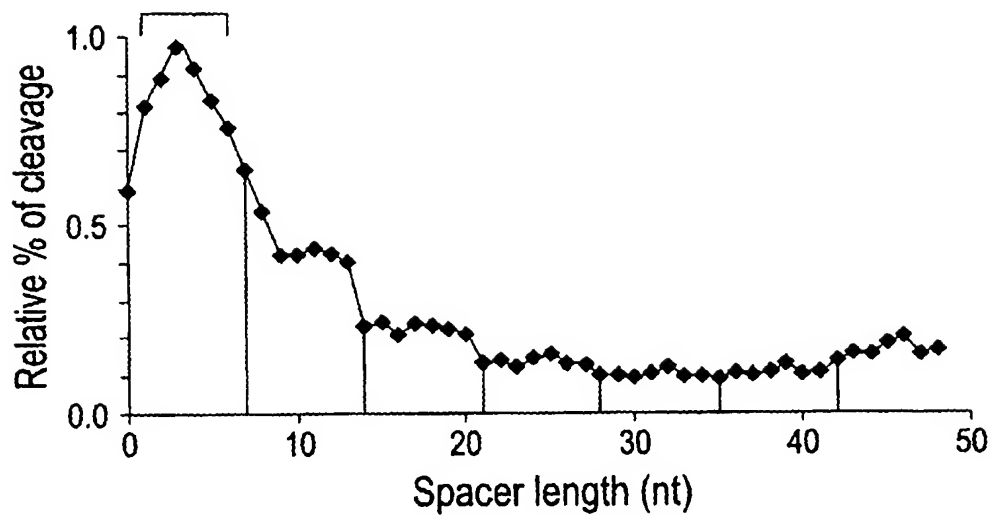
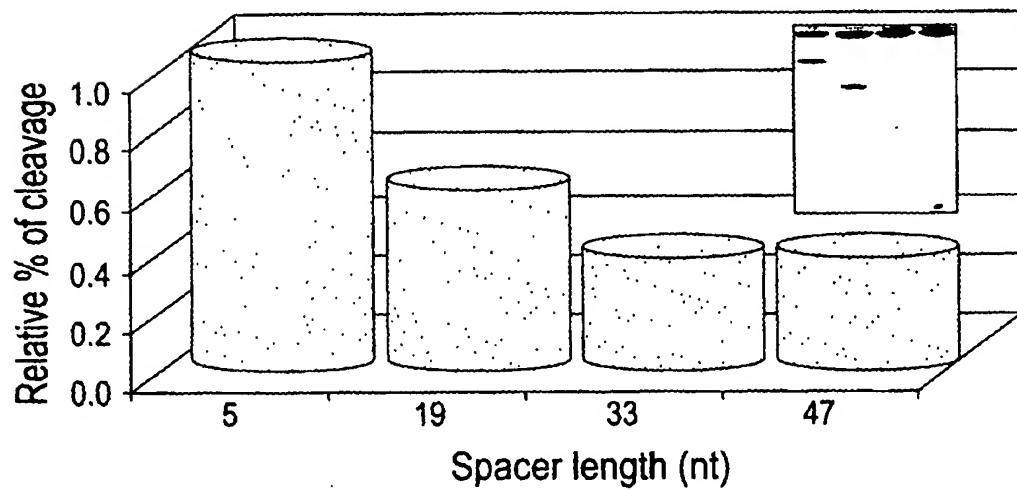
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FIG. 12BFIG. 12C

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FIG. 13AFIG. 13B

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FIG. 13CFIG. 13D

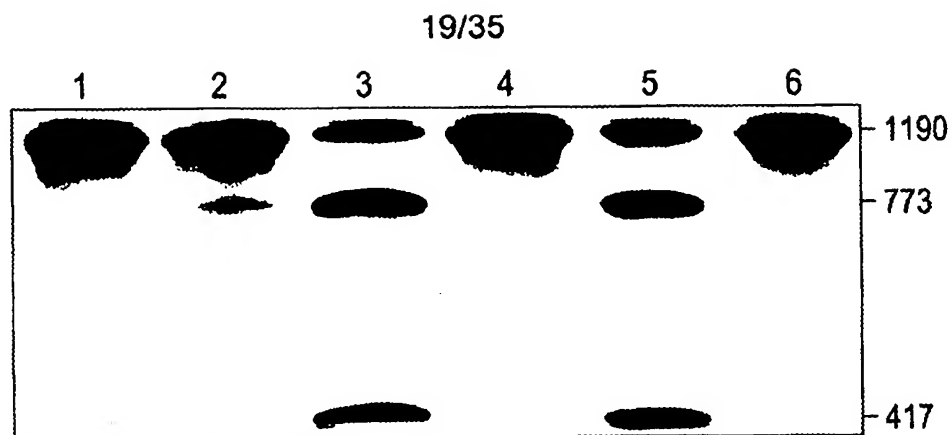


Fig. 14A

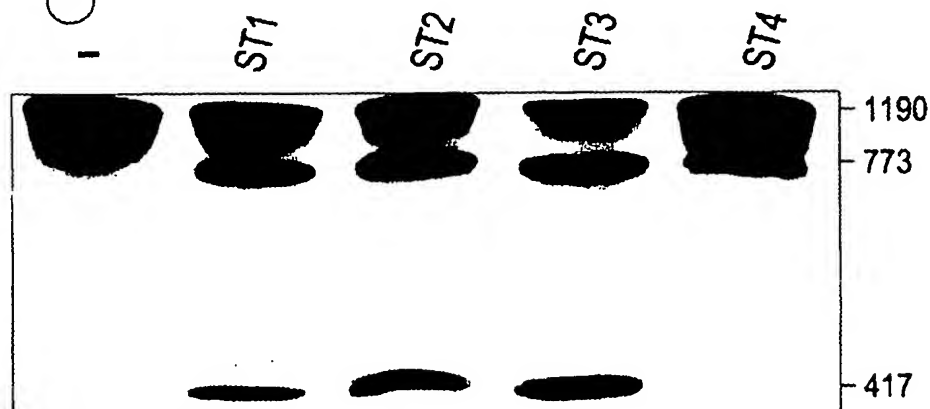
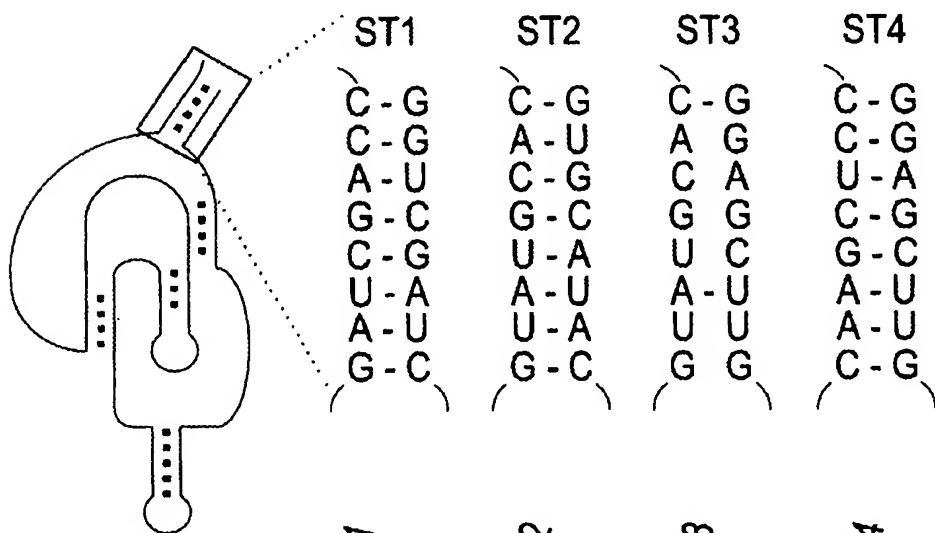
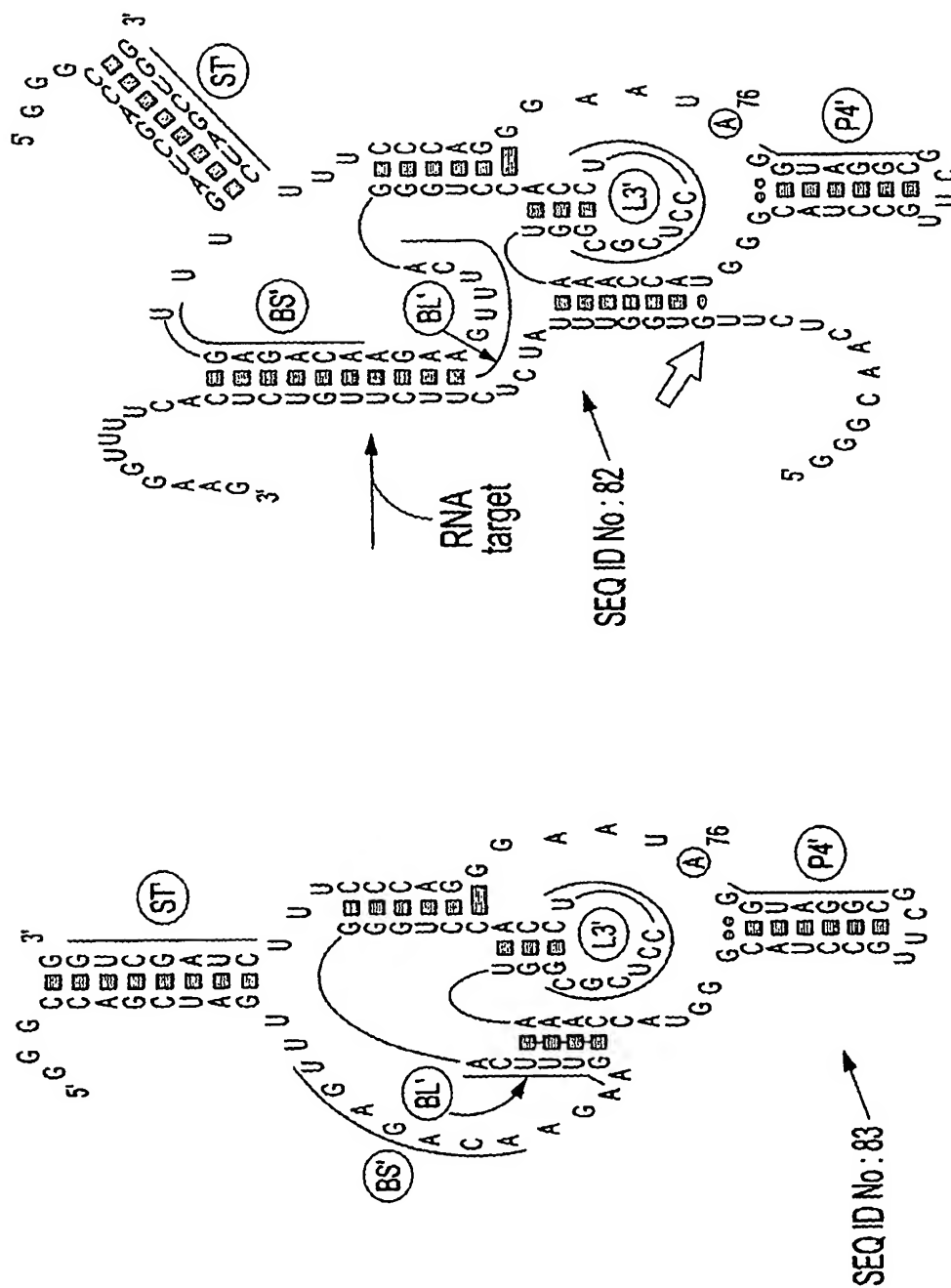


Fig. 14B

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FIG. 15A

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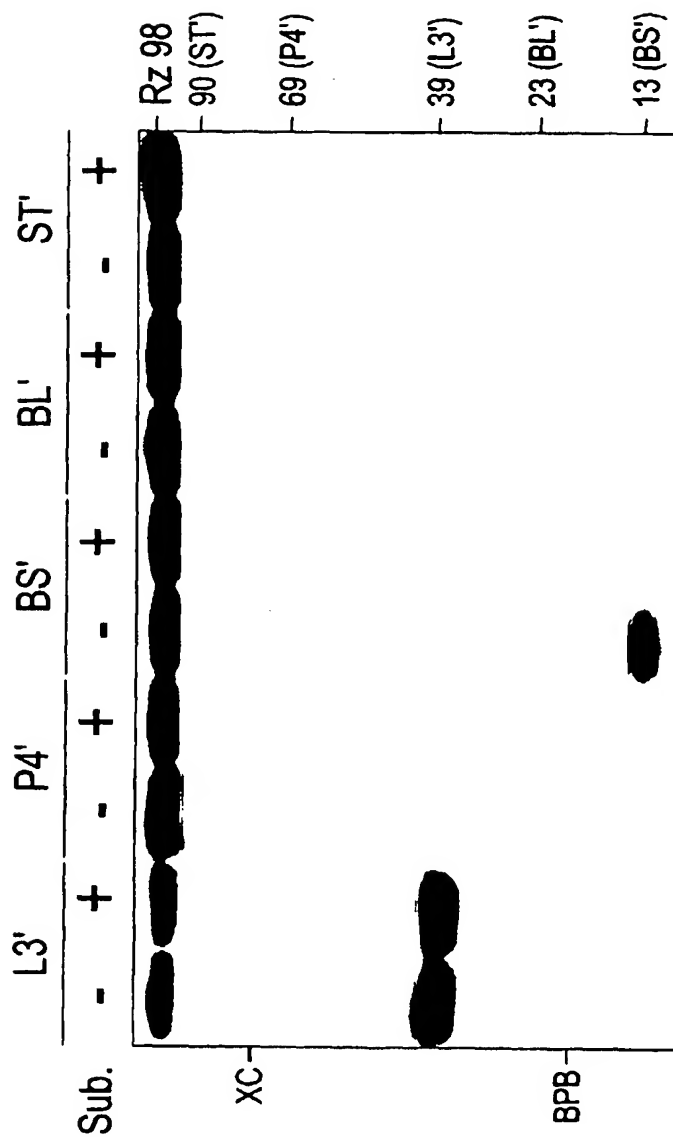
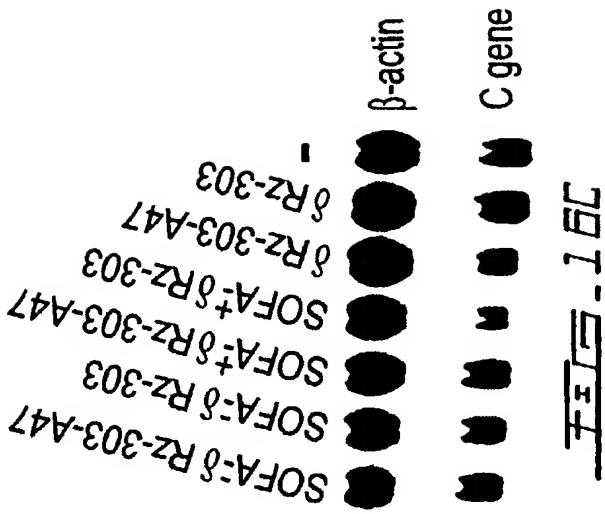
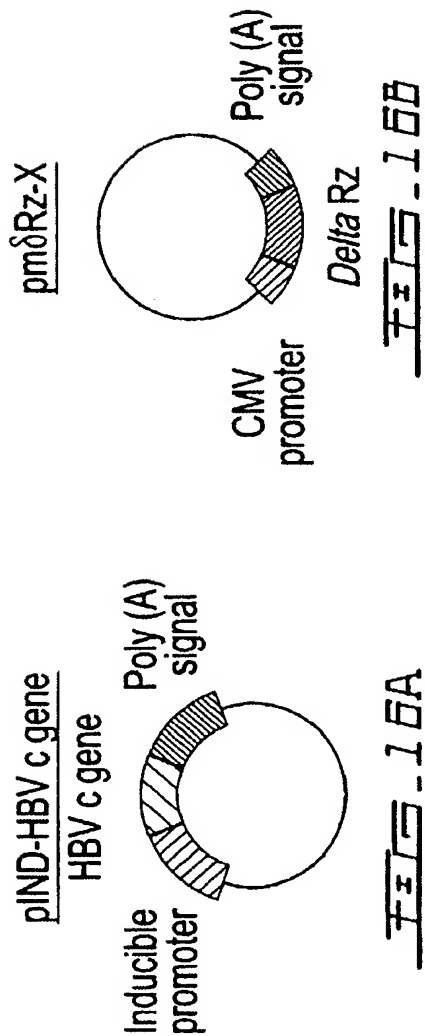
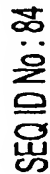


Fig. 15B

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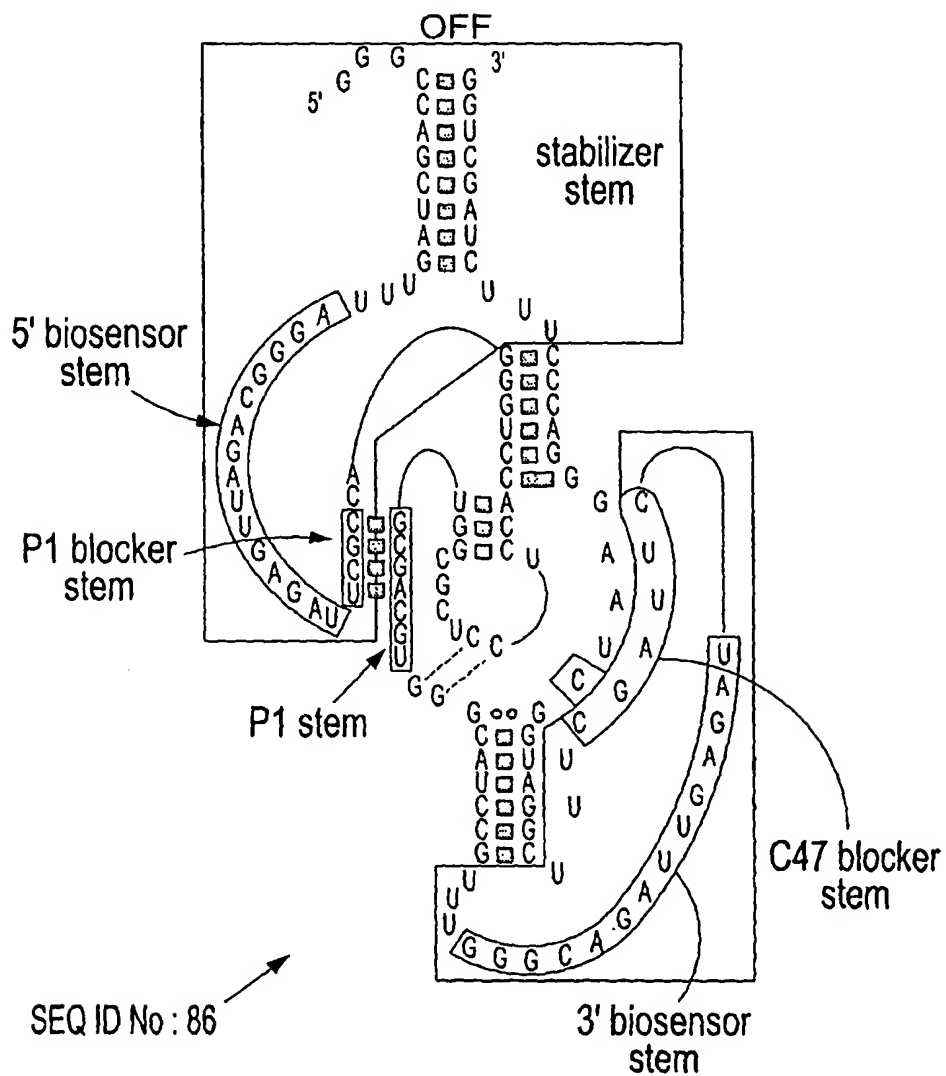


SEQ ID No: 85



FED-17A

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FIG. 17B

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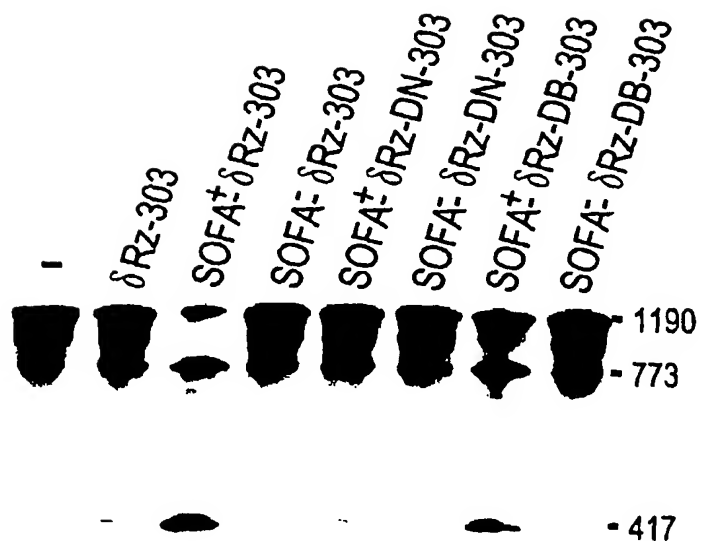


Figure 170

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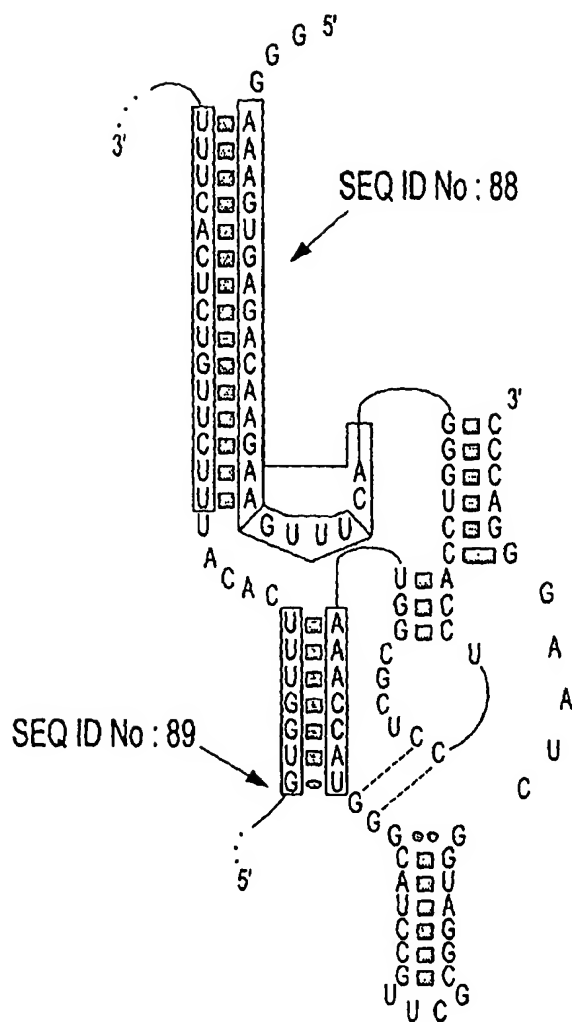


FIG. 18A

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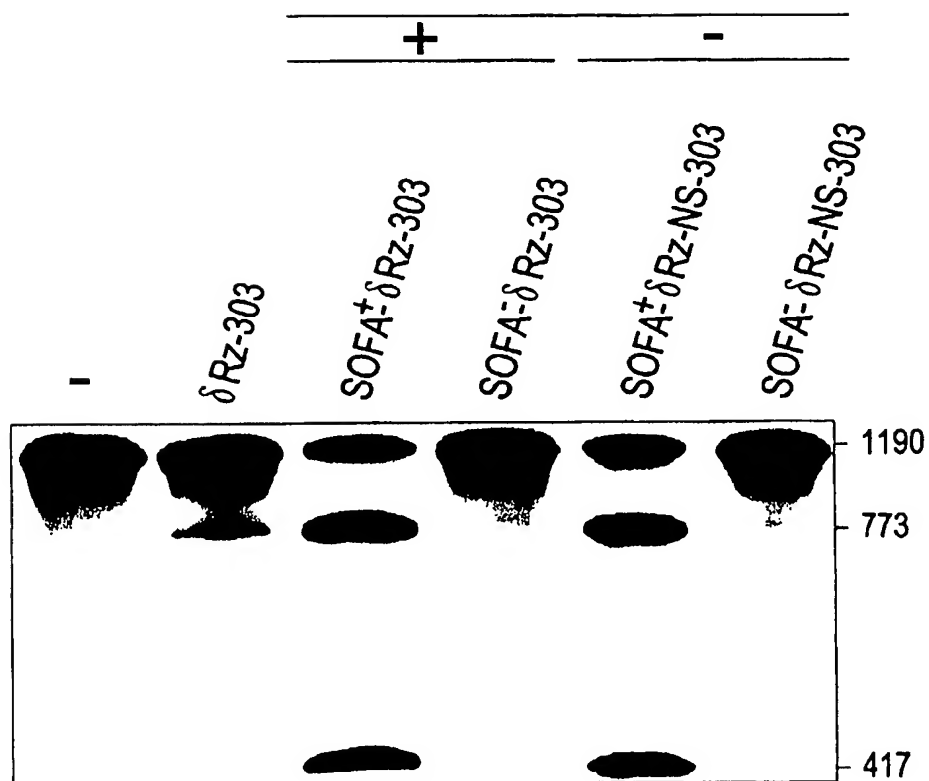


Fig. 1B

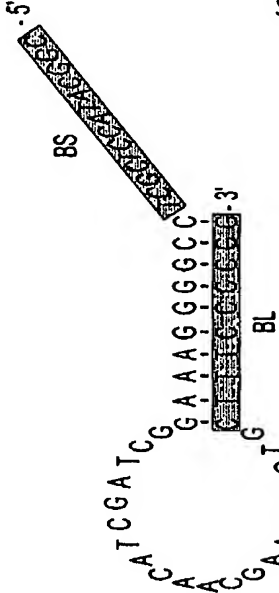
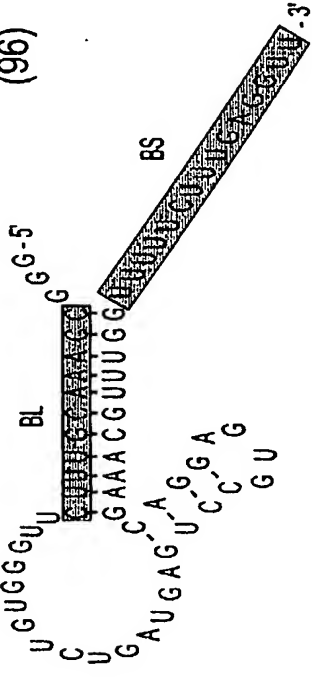
Nucleic acid enzyme	WT	off conformation
<div>DNazyme (Cleavage activity) (SEQ ID No)</div>	<div>3'-TGGTGAAGA AAAGGGCC-5' G A G G C G A T C G A C A T C G (91)</div>	<div> (99)</div>
<div>hammerhead Rz (Cleavage activity) (SEQ ID No)</div>	<div>5'-UUGGUGU ACGUUGG-3' U C A A G G A U G A U C C G A G U (88)</div>	<div> (96)</div>

FIG-19A

Nucleic acid enzyme	on conformation
<div>DNzyme (Cleavage activity)</div> <div>(SEQ ID No)</div>	<div><p>(95)</p></div>
<div>hammerhead Rz (Cleavage activity)</div> <div>(SEQ ID No)</div>	<div><p>(92)</p></div>

FIG-19B

Nucleic acid enzyme	WT	off conformation
hairpin Rz (Cleavage activity)	<p>5'-AAACAG GUCAA-UA-3'</p> <p>A A C-G</p> <p>GA C-G</p> <p>A-U</p> <p>A G-CCA U</p> <p>A A C</p> <p>A C C-GG U A</p> <p>A C C-G U</p> <p>G U</p> <p>(89)</p>	<p>5'-GAGCAUAACGAGCUAAC-3'</p> <p>BS</p> <p>BL</p> <p>A G-A</p> <p>A G-A</p> <p>A G-A</p> <p>A G-A</p> <p>A G-A</p> <p>U G-A</p> <p>CA A-U</p> <p>C-G</p> <p>C-G</p> <p>A-U</p> <p>A G-CCA U</p> <p>A A C</p> <p>A C C-GG U A</p> <p>A C C-G U</p> <p>G U</p> <p>(97)</p>

FIG. 19C

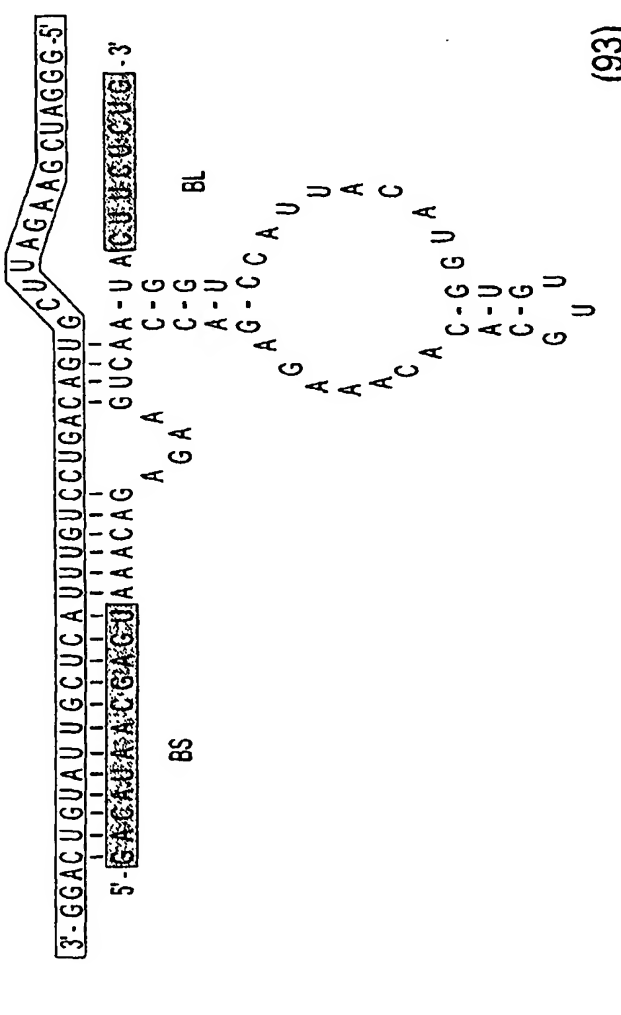
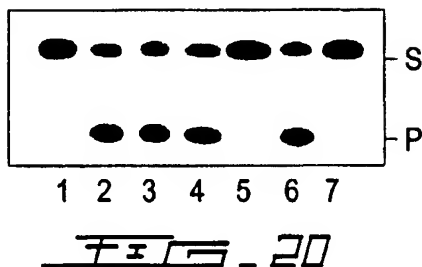
Nucleic acid enzyme	on conformation
hairpin Rz (Cleavage activity) (SEQ ID No)	 <p>(93)</p>

FIG. 190

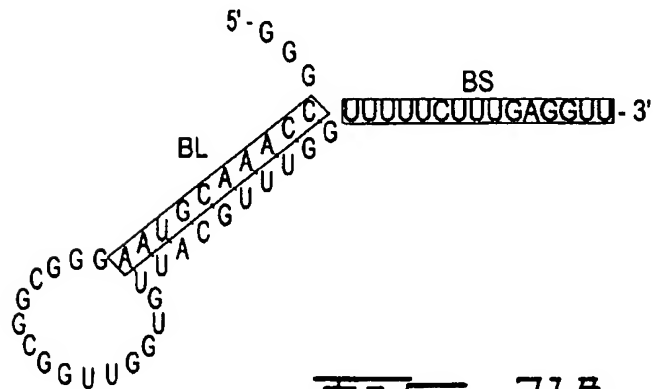
35/35



siRNA

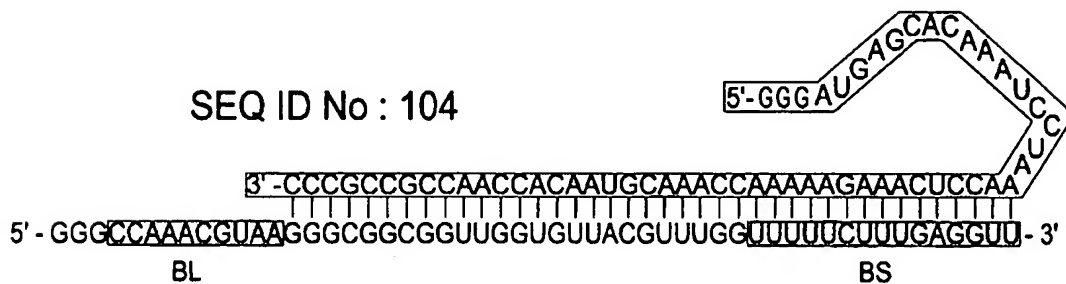
5'-GGGCGGCGGUUGGUGUUACGUUUGG-3'

SEQ ID No : 102

FIS - 21A

SEQ ID No : 103

SEQ ID No : 104



SEQUENCE LISTING

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PERREAULT, Jean-Pierre

BERGERON, Lucien Junior

<120> A TARGET-DEPENDENT NUCLEIC ACID ADAPTER

<130> 6078-18PCT

<150> US 60/585,093

<151> 2004-07-06

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<210> 4

<211> 62

<212> DNA

<213> Artificial Sequence

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<223> antisense primer

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aa 62

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<212> DNA

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<223> Antisense oligonucleotide Rz-down

<220>

<221> misc_feature

<222> 44, 45, 46, 47, 48, 49, 71, 72, 73, 74

<223> n = A,T,C or G

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gtggaccctg nnnn 74

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<211> 67

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<220>

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<222> 33, 34, 35, 36, 37, 38, 39

<223> n = A,T,C or G

<220>

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<222> 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52

<223> n = A,T,C or G, or is absent

<220>

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<222> 53, 54, 55, 56

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61

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<400> 12

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<210> 13

<211> 69

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<400> 13

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<223> substrate h

<400> 14

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<222> (22)...(28)

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<222> (35)...(41)

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<400> 17

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<210> 18

<211> 37

<212> DNA

<213> Artificial Sequence

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<223> reverse primer

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<223> forward primer

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28

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<223> reverse primer

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51

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<210> 38

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42

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<210> 40

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agacugagau

10

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guacucaugc

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10

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auccguucgc ggauggauaa gggacccuuu cuagcugg 98

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<223> Double-SOFA-ribozyme

<400> 86

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gcugg 125

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27

<210> 89

<211> 81

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ucgcggaugg cuaagggacc c 81

<210> 90

<211> 37

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<400> 90

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37

<210> 91

<211> 50

<212> DNA

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<223> Hairpin Rz (cleavage activity) - WT

<400> 91

aaacagagaa gucaaccaga gaaacacacg uugugguaca uuaccuggua 50

<210> 92

<211> 50

<212> DNA

<213> Artificial Sequence

<220>

<223> Hairpin Rz (ligation activity) - WT

<400> 92

aaacagagaa gucaaccaga gaaacacacg uugugguaca uuaccuggua 50

<210> 93

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> DNazyme (cleavage activity) - WT

<400> 93

ccgggggaaag gctagctaca acgaagaagt gct 33

<210> 94

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aaccucaaag aaaaaccaa cguaacacca a

31

<210> 95

<211> 44

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44

<210> 96

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gggucuauugu acaagcacuu cuguuucccc ggagcgaggu augccg

46

<210> 98

<211> 67

<212> DNA

<213> Artificial Sequence

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<223> Hammerhead ribozyme - (cleavage activity)

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ugagguu

67

<210> 99

<211> 70

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<223> Hairpin Rz (cleavage activity)

<400> 99

gacauaacga guaaacagag aagucaacca gagaaacaca cguuguggua cauuaccugg 60
uacuucucug 70

<210> 100

<211> 82

<212> DNA

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<223> Hairpin Rz (ligation activity)

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uacuucucug cgaauucuug au 82

<210> 101

<211> 55

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<223> DNzyme (cleavage activity)

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cggcatacct cgctccgggg aaaggctagc tacaacgaag aagtgccttc cccgg 55

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<223> Si RNA

<400> 102

gggcggcggu ugguguuacg uuugg

25

<210> 103

<211> 53

<212> DNA

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<220>

<223> Si RNA (off)

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53

<210> 104

<211> 59

<212> DNA

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<223> Substrate

<400> 104

gggaugagca caaauccuaa accucaaaga aaaaccaaac guaacaccaa ccgccgcc 59

INTERNATIONAL SEARCH REPORT

International application No.
CT/CA2005/001051

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7): C07H 21/00, C12N 15/55, C12N 9/22, C12N 9/00, C12N 15/11, C07H 21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(7): C07H 21/00, C12N 15/55, C12N 9/22, C12N 9/00, C12N 15/11, C07H 21/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Delphion, Pubmed, Biosis, Caplus, STN Registry (SEQ ID NO 1)

Keywords: ribozyme, switch, blocker, stem, biosensor, riboswitch, TRAP and SOFA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	BERGERON, L.J., et al., "Target-dependent on/off switch increases ribozyme fidelity", Nucleic Acids Research. February 2005, Vol. 33, No. 4, pages 1240-1248. (See whole document)	1 to 40
A	BURKE, D. H., et al., "Allosteric Hammerhead Ribozyme TRAPs", Biochemistry. 2002, Vol. 41, pages 6588-6594. (See abstract and page 6590 and Figure 2)	1 to 40
A	SOUKUP, G. A., et al., "Engineering precision RNA molecular switches", Proceedings of the National Academy of Sciences USA. 1999, Vol. 96, pages 3584-3589. (See whole document.)	1 to 40

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 September 2005 (28-09-2005)

Date of mailing of the international search report

27 October 2005 (27-10-2005)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001051

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WANG, D. Y., et al., "A general approach for the use of oligonucleotide effectors to regulate the catalysis of RNA-cleaving ribozymes and DNAzymes", Nucleic Acids Research. 2002, Vol. 30, No. 8, pages 1735-1742. (See whole document, see Figure 1)	1 to 40
A	ROBERTSON, M. P., et al., "In vitro selection of an allosteric ribozyme that transduces analytes to amplicons", Nature Biotechnology. 1999, Vol. 17, pages 62-66. (See Figure 2)	1 to 40
A	SOUKUP, G. A., et al., "Nucleic acid molecular switches", Trends in Biotechnology. 1999, Vol. 17, No. 12, pages 469-476. (See whole document)	1 to 40
A	SILVERMAN, S. K., et al., "Rube Goldberg goes (ribo)nuclear? Molecular switches and sensors made from RNA", RNA. 2003, Vol. 9, (See pages 377-383 and Figure 3(c).)	1 to 40
A	ROY, G., et al., "Delta ribozyme has the ability to cleave in trans an mRNA", Nucleic Acids Research. 1999, Vol. 27, No. 4, pages 942-948. (See whole document.)	6, 16, 26 and 37